

Complex formation of small ligands with nucleic acids

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“Courage doesn’t always roar. Sometimes courage is the little voice at the end of the day that says I’ll try again tomorrow.” - Mary Anne Radmacher

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Spis treści

Abstract	vi
Streszczenie	viii
List of abbreviations	x
List of figures	xii
List of tables	xx
1 Introduction	1
1.1 Nucleic acids	1
1.1.1 DNA	1
1.1.2 RNA	6
1.1.3 Messenger RNA	11
1.1.4 Transfer RNA	14
1.1.5 Ribosomal RNA	19
1.1.6 Influence of intercalation on DNA and RNA	23
1.2 Cyanine dyes YO-PRO-1 and YOYO-1	30
1.3 Anthracyclines	38
1.4 Fluorescence correlation spectroscopy	57
1.5 Brightness Analysis Method	67
1.6 Continuous fluorescence photobleaching	77
2 Materials and methods	85
2.1 Materials	85
2.1.1 Buffers	85
2.1.2 Nucleic acids	85
2.1.3 Dyes and anthracyclines	86
2.2 Methods	86
2.2.1 FCS set-up	86
2.2.2 FCS measurements	86
2.2.3 Calibration	87
2.2.4 Brightness method samples	89
2.2.5 Brightness model	89
2.2.6 Polarisation experiments	91
2.2.7 YO-PRO-1 target determination	94
3 Results and discussion	96
3.1 Equilibrium constant determination	96
3.1.1 Optimisation of laser power	97
3.1.2 Mechanisms of studied reactions	98
3.1.3 Equilibrium constant determination	119
3.2 Non-fluorescent complexes fraction determination	134
3.2.1 Determination of experimental and measurement parameters in 5% agarose gel	136

3.2.2	Determining the fraction of emitting DNA-YO-PRO-1 complexes immobilised in 5% agarose gel	139
3.3	YO-PRO-1 dye targets determination	149
4	Main results, conclusions and future prospects	154
5	Bibliography	170

Abstract

Progress in molecular biology depends on discoveries of new specific dyes that stain cellular structures. Observing intracellular components, studying their interactions, and quantifying them will improve our understanding of cellular life processes. This knowledge will enable the development of new therapeutic methods that directly target cellular structures and improve existing ones.

Today, fluorescent dyes are mainly used to investigate cellular targets. They are safer and easier to use than the radioactive tracers used in the past, and they offer higher selectivity. The application of fluorescent dyes enables the study of nucleic acid interactions, determination of protein concentration, structure, and interactions, determination of ion concentrations in the cell, and observation of ion channel function, as well as of whole organelles. Many disorders or abnormalities in the concentrations, structures, or interactions of these biomolecules are markers of numerous diseases. Fluorescent dyes also allow the researcher to observe, with good resolution, many different cell components of interest.

In my studies, I used fluorescent dyes and anthracyclines to investigate their reactions with nucleic acids. The experimental part of my dissertation is divided into four sections: determination of cyanine dye equilibrium constants; determination of anthracycline equilibrium constants; photobleaching of the YO-PRO-1 dye complexed to immobilised DNA; and determination of YO-PRO-1 dye's cellular targets. In the next paragraphs, I briefly describe sections of my experimental work.

First, I have focused on cyanine dyes that stain nucleic acids. Using this group, I studied the interactions between the YO-PRO-1 and YOYO-1 dyes at the single-molecule level. In my work, I carried out a quantitative analysis of their interactions with nucleic acids. I determined the equilibrium constant and investigated the mechanism of interaction between the YOYO-1 dye and DNA.

The second area of my interest was anthracyclines, as they are also intercalators. They are a family of antibiotics initially isolated from *Streptomyces* bacteria with a broad spectrum of activity in oncological treatment. These drugs intercalate into the DNA in the cell's nucleus, destabilising its structure and interacting with Topoisomerase II. These changes prevent transcription, leading the cell to enter the apoptotic cycle. Due to the weakened DNA repair mechanisms in cancer cells, they are more sensitive to the effects of anthracyclines.

However, these chemotherapeutics are characterised by severe cardiotoxic effects. Numerous studies are underway to develop new anthracycline derivatives with high therapeutic efficacy while reducing cardiotoxicity. Numerous clinical trials using anthracyclines are reported in the literature. Despite extensive attempts to modify this group of antibiotics, anthracyclines remain a highly toxic group of highly effective anticancer drugs.

In my work, I investigated the binding of anthracyclines to DNA and determined the equilibrium constants for the complex-formation reactions.

I also utilised a new method for characterising biochemical reactions by determining their equilibrium constants, which involves analysing changes in molecular brightness. This technique, using fluorescence, allows the equilibrium constant for the formation of non-covalent complexes at nanomolar concentrations, like those found in cells, to be accurately determined. The method is based on the change

in intrinsic molecular brightness that results from the complexation reaction. It also enables research into the reaction mechanism.

In my work, I use this technique to determine the equilibrium constants for the complexation of the dyes YOYO-1 and YO-PRO-1, and of anthracyclines with DNA. Moreover, applying this method has also allowed me to determine the fraction of dyes bound to immobilised DNA that do not undergo excitation due to the orientation of their dipole moments.

Also, I identified the cellular targets of YO-PRO-1 by testing various nucleic acid probes in buffer. The targets were further confirmed in HeLa cells by my colleague.

Streszczenie

Postęp w biologii molekularnej uzależniony jest od odkrywania nowych specyficznych barwników barwiących struktury komórkowe. Obserwacje komponentów wewnątrzkomórkowych, badanie ich oddziaływań, a także możliwość pomiaru i określenia tych interakcji w sposób ilościowy pozwalają zrozumieć procesy życiowe w komórce. Wiedza ta pozwoli na utworzenie nowych, a także ulepszenie istniejących metod terapeutycznych celujących bezpośrednio w struktury obecne w komórce.

Obecnie zastosowanie znajdują głównie barwniki fluorescencyjne. Są one bezpieczniejsze i łatwiejsze w użyciu od stosowanych w przeszłości znaczników radioaktywnych, a także cechują się większą selektywnością. Zastosowanie barwników fluorescencyjnych pozwala na badanie interakcji kwasów nukleinowych, określenie stężeń, struktur oraz interakcji białek, wyznaczenie stężenia jonów w komórce, obserwację pracy kanałów jonowych czy nawet całych organelli. Wiele zaburzeń czy nieprawidłowości w stężeniach, strukturze bądź oddziaływaniach tych biomolekuł jest markerem licznych chorób. Barwniki fluorescencyjne umożliwiają również z dobrą rozdzielczością obserwację wielu różnych interesujących badacza komponentów komórki.

W moich badaniach skupiłam się na badaniach barwników cyjaninowych i antracyklinach, badając ich reakcje z kwasami nukleinowymi. Część eksperymentalną mojej pracy doktorskiej podzieliłam na 4 sekcje: wyznaczanie stałych równowagi barwników cyjaninowych, wyznaczanie stałych równowagi antracyklin, wypalanie barwnika YO-PRO-1 kompleksującego immobilizowane DNA oraz wyznaczenie w buforze komórkowych targetów barwnika YO-PRO-1. W kolejnych akapitach pokrótce opiszę wspomniane sekcje części eksperymentalnej mojej pracy doktorskiej.

W swojej pracy skupiłam się na barwnikach cyjaninowych, stosowanych do barwienia kwasów nukleinowych. Zastosowanie przedstawicieli tej grupy, barwników YO-PRO-1 i YOYO-1, pozwoliło mi na badanie ich interakcji na poziomie pojedynczych molekuł. W swojej pracy przeprowadziłam ilościową analizę ich interakcji z kwasami nukleinowymi. Wyznaczyłam stałe równowagi dla reakcji tych barwników z DNA, a także zbadałam mechanizm interkalacji barwnika YOYO-1.

Do grupy interkalatorów zalicza się również antracykliny. Jest to grupa antybiotyków, pierwotnie wyizolowana z bakterii *Streptomyces*, o szerokim spektrum działania w leczeniu onkologicznym. Leki te interkalują w DNA w jądrze komórki, destabilizując jego strukturę, a także oddziałując na topoizomerazę II. Zmiany te uniemożliwiają proces transkrypcji, w wyniku czego komórka wchodzi w cykl apoptyczny. Ze względu na osłabione mechanizmy naprawcze DNA w komórkach nowotworowych są one bardziej wrażliwe na działanie antracyklin.

Chemioterapeutyki te cechuje jednak silne działanie kardiotoksyczne. Prowadzone są liczne badania, mające na celu syntezę nowych pochodnych antracyklin, z zachowaniem wysokiego efektu terapeutycznego przy jednoczesnym obniżeniu kardiotoksyczności. W literaturze opisywane są liczne badania kliniczne, w których stosowane były antracykliny. Pomimo licznych prób modyfikacji tej grupy antybiotyków, antracykliny pozostają bardzo toksyczną grupą wysoce efektywnych leków przeciwnowotworowych.

W swojej pracy skupiłam się na zbadaniu wiązania antracyklin z DNA oraz wyznaczaniu stałych równowagi reakcji tworzenia kompleksów.

Nową metodą służącą do charakteryzacji reakcji biochemicznych poprzez wyznaczanie stałych równowagi tych reakcji jest analiza zmian jasności molekularnej. Technika ta, dzięki zastosowaniu fluorescencji, pozwala przy stężeniach nanomolarnych, a więc stężeniach charakteryzujących związki w komórkach, z dobrą dokładnością wyznaczyć stałą równowagi tworzenia kompleksów niekowalencyjnych. Metoda ta opiera się na zmianie wewnętrznej jasności molekularnej, która wynika z reakcji kompleksowania. Umożliwia ona również badania nad mechanizmem reakcji.

W swojej pracy szeroko korzystam z tej techniki, wyznaczając stałe równowagi kompleksowania barwników YOYO-1 i YO-PRO-1 oraz antracyklin z DNA. Zastosowanie tej metody pozwoliło mi również na określenie frakcji barwników związanych z immobilizowanym DNA, które wskutek orientacji swojego momentu dipolowego nie ulegają wzbudzeniu.

Dodatkowo określiłam w buforze targety komórkowe barwnika YO-PRO-1, co zostało potwierdzone przez inne osoby w projekcie we wnętrzu komórek linii komórkowej HeLa.

List of abbreviations

aaRS - aminoacyl-tRNA synthetase

aa-tRNA - aminoacyl-tRNA

ACF - autocorrelation function

ACQ - aggregation-caused quenching

ATP - adenosine triphosphate

BAM - brightness analysis method

cDNA - complementary DNA

CFP - continuous fluorescence photobleaching

circRNA - circular ribonucleic acid

CPM - counts per molecule

DAU - daunorubicin

DNA - deoxyribonucleic acid

DOX - doxorubicin

DSB - double-strand break

EPI - epirubicin

ETS - external transcribed spacer

FCCS - fluorescence cross-correlation spectroscopy

FCS - fluorescence correlation spectroscopy

FIDA - fluorescence intensity distribution analysis item **FLCS** - fluorescence lifetime correlation spectroscopy

FRAP - fluorescence recovery after photobleaching

FRET - Förster Resonance Energy Transfer

IDA - idarubicin

IRES - internal ribosome entry sites

ISC - intersystem crossing

ITC - isothermal titration calorimetry

ITS - internal transcribed spacer

lncRNA - long noncoding ribonucleic acid

LSU - large ribosomal subunit
MB - molecular brightness
MBA - molecular brightness analysis
mRNA - messenger ribonucleic acid
miRNA - micro ribonucleic acid
NADH - nicotinamide adenine dinucleotide + hydrogen
ncRNA - noncoding ribonucleic acid
NORs - nucleolar organising regions
PCH - photon counting histogram
PDA - photon distribution analysis
Pol I - polymerase I
PSF - point spread function
rDNA - ribosomal DNA
RNA - ribonucleic acid
RNAi - ribonucleic acid interference
RNP - ribonucleoprotein
ROS - reactive oxygen species
RPs - ribosomal proteins
rRNA - ribosomal ribonucleic acid
siRNA - small interfering ribonucleic acid
snRNA - small nuclear ribonucleic acid
snRNP - small nuclear small nuclear ribonucleoprotein
SPAD - single-photon avalanche diode
SSB - single-strand break
SSU - small ribosomal subunit
STED-FCS - stimulated emission depletion FCS
tRFs - tRNA-derived fragments
tRNA - transfer ribonucleic acid
UTRs - untranslated regions

List of figures

Figure 1: Scheme of DNA helix structure and base pairing in the DNA structure [2].

Figure 2: Watson–Crick base pairs exhibit specific hydrogen bonding patterns: (a) adenine–thymine (A–T) pairs, and (b) guanine–cytosine (G–C) pairs. The key atoms involved in hydrogen bonding include nitrogen (N), oxygen (O), and hydrogen (H). Carbon atoms remain unlabelled for clarity. Hydrogen bonds stabilise the base pairing and are denoted by dotted lines. Partial electric charges, represented as + and -, highlight the polar nature of these bonds, typically amounting to about one-third of the charge of an electron or proton. [3].

Figure 3: A scheme of base pairs consecutive components with given distances [3].

Figure 4: The sugar-phosphate backbone of DNA can be visualised as helically wrapped chains around an imaginary central cylinder. Two distinct representations highlight this structure: (a) the sugar rings are depicted as shaded or filled circles, emphasising their position within the helix. The phosphate groups connecting the sugars are shown as thin lines, outlining the structural backbone. (b) The phosphate groups are represented as open circles, while the sugar rings are drawn as thin lines. This view shifts focus to the positioning of phosphates along the helix. The helical arrangement of the sugar-phosphate backbone is critical for DNA’s stability and function [3].

Figure 5: Overview of RNA Primary and Secondary Structure and Base Pairing Patterns. (A) The nucleotide building blocks of RNA primary structure consist of a ribose sugar, which contains a 2’ hydroxyl group unique to RNA compared to DNA; phosphodiester groups that connect the 5’ and 3’ hydroxyl groups of ribose sugars; and the nucleobases adenine, guanine, uracil, and cytosine, which define the RNA sequence. (B) Canonical Watson–Crick–Franklin hydrogen bonding patterns, with dashed lines indicating hydrogen bonds. (C) An A-form RNA duplex secondary structure. Red dots denote metal ions, yellow dashes highlight electrostatic interactions such as hydrogen bonds, and black dashes indicate stacking interactions. (D) Representative canonical RNA secondary structures from the 5’ end of the long non-coding RNA (lncRNA) B2-SINE. Single and double solid lines represent A–U and G–C Watson–Crick–Franklin base pairs, respectively, while circles denote wobble base pairs. [23].

Figure 6: tRNA structure. (A) Two-dimensional cloverleaf representation of tRNA. From the 5’ to the 3’ end, the structural elements include the acceptor stem (green), D-arm (pink), anticodon arm (blue), which contains the anticodon (highlighted in darker blue), the variable arm (orange), and the T-arm (purple). The discriminator base (position 73, shown in yellow) lies unpaired at the 3’ end, immediately before the terminal CCA residues. Aminoacyl-tRNA synthetases catalyse the attachment of an amino acid to the terminal 3’ adenosine. Numbered positions indicate conserved bases. (B) Three-dimensional structure of yeast tRNA^{Phe}. The same colour scheme as in panel (A) is used to highlight the structural regions. Intramolecular interactions between the D-arm (pink)

and the T-arm (purple) form the elbow region, a critical feature that facilitates proper tRNA folding into its characteristic L-shaped structure. [56]

Figure 7: 2D structure of YO-PRO-1 dye. The dye consists of benzoxazole and quinoline rings connected with a cyanine-based linker. The green area marks the aromatic structure responsible for fluorescence.

Figure 8: Scheme of YO-PRO-1-DNA complex formation. YO-PRO-1 intercalates between base pairs in the DNA helix. The binding size is 5 bp. After complex formation, the non-fluorescent dye (in a free state) emits green fluorescence ($\lambda = 509$ nm).

Figure 9: 2D structure of YOYO-1 dye. The dye consists of benzoxazole and quinoline rings connected with a cyanine-based linker. The dye has two aromatic moieties. The green area marks the aromatic structure responsible for fluorescence.

Figure 10: Scheme of YOYO-1-DNA complex formation. YOYO-1 intercalates into the DNA helix in between base pairs. The binding size is 3.5 bp. After complex formation, the non-fluorescent dye (in a free state) emits green fluorescence ($\lambda = 509$ nm).

Figure 11: 2D structure of doxorubicin. The red area marks the aromatic structure responsible for fluorescence.

Figure 12: 2D structure of daunorubicin. The red area marks the aromatic structure responsible for fluorescence.

Figure 13: 2D structure of idarubicin. The red area marks the aromatic structure responsible for fluorescence.

Figure 14: 2D structure of epirubicin. The red area marks the aromatic structure responsible for fluorescence.

Figure 15: Scheme of DOX-DNA complex formation. Doxorubicin intercalates into the DNA helix between base pairs. In excess of doxorubicin, the second complex occurs; additional doxorubicin molecules create a complex with the DOX-DNA complex. The mechanism of the reaction is analogous for daunorubicin, idarubicin and epirubicin.

Figure 16: Principle of fluorescence correlation spectroscopy (FCS). Fluorescently labelled particles diffuse through the confocal detection volume, producing intensity fluctuations over time. Autocorrelation analysis of the fluorescence trace yields the correlation function $G(\tau)$. The characteristic decay of $G(\tau)$ (solid curves) reflects the particle mobility, while the inverse of the amplitude at zero lag, $G(0)$, is proportional to the particle concentration (dashed curves). [283]

Figure 17: A Jablonski diagram of a fluorophore in a three-level system illustrates its photophysical pathways. Upon excitation, the molecule transitions from the ground state (S_0) to the excited singlet state (S_1). From S_1 , the excess energy can be released through several competing processes, each defined by a specific

rate constant (k). These include fluorescence emission, internal conversion, or intersystem crossing to the triplet state (T_1). Importantly, photobleaching primarily occurs from this long-lived triplet state (T_1), as the molecule in this state is more prone to undergo photochemical reactions that irreversibly destroy its fluorescence. [368]

Figure 18: The plot illustrates an example of FCS autocorrelation curves acquired during calibration (in a buffer environment, 25°C). Each autocorrelation curve was measured over 20 seconds. The data were fitted using a one-component diffusion model that accounts for the contribution from the triplet state (as per Equation 1). Experimental data points are depicted in red, while the green line represents the autocorrelation function resulting from the fitting process. In the plot, there are 6 separate autocorrelation curves, with the fitting curve. Additionally, the bottom panel displays the residual curve, which denotes the disparity between the experimental and fitted data points.

Figure 19: The plot illustrates an example of FCS autocorrelation curves acquired during calibration (in a buffer environment, 36°C). Each autocorrelation curve was measured over 20 seconds. The data were fitted using a one-component diffusion model that accounts for the contribution from the triplet state (as per Equation 1). Experimental data points are depicted in red, while the green line represents the autocorrelation function resulting from the fitting process. In the plot, there are 6 separate autocorrelation curves, with the fitting curve. Additionally, the bottom panel displays the residual curve, which denotes the disparity between the experimental and fitted data points.

Figure 20: Scheme of the optical pathway. A laser pulse is polarised in the polarisation module before reaching the sample in the microscope. The fluorescence signal coming from the sample is then detected by two SPAD detectors and transferred to the computer for analysis in the Syphotime program.

Figure 21: Scheme of the λ -DNA (green) in focal volume (orange). The cylinder approximation (purple) was used to calculate the DNA volume within the focal volume.

Figure 22: Collected fluorescence for measured samples upon increased applied laser power. PBS, and YO-PRO-1 and YOYO-1 dyes in a free state, exhibit no fluorescence properties; collected photons are connected with a dispersion of the laser beam on molecules present in the focal volume. The fluorescent complex of DNA (100 nM) and YO-PRO-1 (20 nM) displays linear response in fluorescence intensity upon laser power increase up to 30 μ W, while for the DNA (2 nM) and YOYO-1 (20 nM) complex, linear response is observed up to 50 μ W. Beyond these thresholds, the complexes undergo photobleaching during measurement.

Figure 23: Relation of the fluorescence intensity and DNA (or tRNA) concentration added to the YO-PRO-1 or YOYO-1 dye. The fluorescence is coming from the excited complexes formed between nucleic acid and the dye. The data were presented in the range 0-50 nM of nucleic acids for better visualisation. Data points are represented with error bars. The pink points ($\blacktriangledown$) correspond to 70 nM YO-PRO-1 bound to DNA at 25°C, the red points ($\blacktriangleleft$) to 20 nM YOYO-1

bound to DNA at 25°C, the blue points (▲) to 40 nM YO-PRO-1 bound to DNA at 36°C, the green (►) points to 20 nM YOYO-1 bound to DNA at 36°C, and the brown points (●) to 40 nM YO-PRO-1 bound to tRNA at 36°C. Along the x-axis, DNA (or tRNA) concentration ranges from 0 to 50 nM, while the y-axis shows fluorescence intensities up to 70,000 counts/s.

Figure 24: The figure presents a log-log plot of molecular brightness (MB, in counts/s per molecule) versus the parameter R (ratio of nucleic acids and dyes concentration). The data points, shown with error bars, correspond to different dye-nucleic acid complexes and experimental conditions. The pink points (▼) correspond to 70 nM YO-PRO-1 bound to DNA at 25°C, the red points (◄) to 20 nM YOYO-1 bound to DNA at 25°C, the blue points (▲) to 40 nM YO-PRO-1 bound to DNA at 36°C, the green (►) points to 20 nM YOYO-1 bound to DNA at 36°C, and the brown points (●) to 40 nM YO-PRO-1 bound to tRNA at 36°C. Straight lines are fitted to each dataset to capture the overall scaling behaviour. The y-axis spans from 10^2 to 10^4 counts/s per molecule, while the x-axis covers R values from approximately 10^{-2} to 1.

Figure 25: Fluorescence titration of anthracyclines with DNA (69 bp, 36°C). Fluorescence intensity (counts/s) of 100 nM solutions of four anthracycline drugs – daunorubicin (DAU, red ◄), doxorubicin (DOX, pink ▼), idarubicin (IDA, blue ►), and epirubicin (EPI, green ▲) – is plotted against increasing DNA concentration (nM). All four dyes exhibit a decrease in fluorescence intensity as more DNA is added, consistent with fluorescence quenching upon drug-DNA binding. Epirubicin shows the highest fluorescence across the titration range, whereas idarubicin gives the lowest signal, with doxorubicin and daunorubicin intermediate. Error bars denote experimental variability.

Figure 26: The figure presents the molecular brightness (MB, counts/s per molecule) of four anthracyclines — daunorubicin (DAU, red ◄), doxorubicin (DOX, pink ▼), idarubicin (IDA, blue ►), and epirubicin (EPI, green ▲) — as a function of the binding ratio R with 69 bp DNA at 36°C. Both axes are plotted on logarithmic scales. Data points are shown with error bars, and fitted lines illustrate the overall trends. The y-axis spans from 10^1 to 10^3 counts/s per molecule, while the x-axis ranges from approximately 10^{-2} to 1.

Figure 27: Exemplary fit of equilibrium constant determination of DNA-YO-PRO-1 complex formation, at 25°C. Pink ▼ represents data points, indicating the count rate acquired for various DNA concentrations added to a 70 nM YO-PRO-1 solution in a titration experiment. The pink line corresponds to the fit of Equation (15). In the fit, $C_{\text{YO-PRO}}$ represents the fitted value of the initial dye concentration, K is the equilibrium constant for the reaction, α is a molecular brightness of the free dye, and γ is a molecular brightness of the formed complex.

Figure 28: Exemplary fit of equilibrium constant determination of DNA-YOYO-1 mono-intercalated complex formation, at 25°C, in equilibrium state. Red ◄ represents data points, indicating the count rate acquired for various DNA concentrations added to a 20 nM YOYO-1 solution in a titration experiment. The red line corresponds to the fit of Equation (15). In the fit, C_{YOYO} represents the

fitted value of the initial dye concentration, and K_1 is the equilibrium constant for the mono-intercalation reaction.

Figure 29: Exemplary fit of equilibrium constant determination of DNA-YOYO-1 bis-intercalated complex formation, at 25°C, in equilibrium state. Red ◀ represents data points, indicating the count rate acquired for various DNA concentrations added to a 20 nM YOYO-1 solution in a titration experiment. The red line corresponds to the fit of Equation (20). In the fit, C_A represents the fitted value of the initial dye concentration, K_1 is the equilibrium constant for the mono-intercalation reaction (treated as constant), K_2 is the equilibrium constant for the bis-intercalation reaction, and n denotes the binding site size.

Figure 30: Exemplary fit of equilibrium constant determination of DNA-YOYO-1 mono-intercalated complex formation, at 36°C, in equilibrium state. Green ▶ represents data points, indicating the count rate acquired for various DNA concentrations added to a 40 nM YOYO-1 solution in a titration experiment. The green line corresponds to the fit of Equation (15). In the fit, C_{YOYO} represents the fitted value of the initial dye concentration, K_1 is the equilibrium constant for the mono-intercalation reaction, and n denotes the size of a binding site.

Figure 31: Exemplary fit of equilibrium constant determination of DNA-YOYO-1 bis-intercalated complex formation, at 36°C, in equilibrium state. Green ▶ represents data points, indicating the count rate acquired for various DNA concentrations added to a 40 nM YOYO-1 solution in a titration experiment. The green line corresponds to the fit of Equation (20). In the fit, C_A represents the fitted value of the initial dye concentration, K_1 is the equilibrium constant for the mono-intercalation reaction (treated as constant), K_2 is the equilibrium constant for the bis-intercalation reaction, and n denotes the binding site size.

Figure 32: Exemplary fit of equilibrium constant determination of DNA-YO-PRO-1 complex formation, at 36°C. Blue ▲ represents data points, indicating the count rate acquired for various DNA concentrations added to a 40 nM YO-PRO-1 solution in a titration experiment. The blue line corresponds to the fit of Equation (15). In the fit, C_{YO-PRO} represents the fitted value of the initial dye concentration, K is the equilibrium constant for the reaction, and α and γ denote the molecular brightnesses of the free dye and the formed complex, respectively.

Figure 33: Exemplary fit of equilibrium constant determination of tRNA-YO-PRO-1 complex formation, at 36°C, in equilibrium state. Brown circles represent data points showing the count rate for various tRNA concentrations added to a 40 nM YO-PRO-1 solution in a titration experiment. The brown line corresponds to the fit of Equation (15). In the fit, C_{YO-PRO} represents the fitted value of the initial dye concentration, and K is the equilibrium constant for the reaction. The data are calculated per concentration of molecules, while α and γ are the molecular brightnesses of the free dye and the formed complex, respectively.

Figure 34: Exemplary fit of equilibrium constant determination of DNA-DOX complex formation, at 36°C, in equilibrium state. Pink ▼ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM DOX solution in a titration experiment. The pink line corresponds to the fit

of Equation (15). In the fit, C_{DOX} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction, while α and γ are molecular brightnesses of the free DOX and DNA-DOX complex, respectively.

Figure 35: Exemplary fit of equilibrium constant determination of (DNA-DOX)-DOX complex formation, at 36°C, in equilibrium state. Pink ▼ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM DOX solution in a titration experiment. The pink line corresponds to the fit of Equation (20). In the fit, C_A represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction (treated as constant), K_2 is the equilibrium constant for the (DNA-DOX)-DOX complex formation reaction, and n denotes the binding site size.

Figure 36: Exemplary fit of equilibrium constant determination of DNA-DAU complex formation, at 36°C, in equilibrium state. Red ◀ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM DAU solution in a titration experiment. The red line corresponds to the fit of Equation (15). In the fit, C_{DAU} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction, while α and γ are molecular brightnesses of the free DAU and DNA-DAU complex, respectively.

Figure 37: Exemplary fit of equilibrium constant determination of (DNA-DAU)-DAU complex formation, at 36°C, in equilibrium state. Red ◀ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM DAU solution in a titration experiment. The red line corresponds to the fit of Equation (20). In the fit, C_A represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction (treated as constant), K_2 is the equilibrium constant for the (DNA-DAU)-DAU complex formation reaction, and n denotes the binding site size.

Figure 38: Exemplary fit of equilibrium constant determination of DNA-IDA complex formation, at 36°C, in equilibrium state. Blue ▲ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM IDA solution in a titration experiment. The blue line corresponds to the fit of Equation (15). In the fit, C_{IDA} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction, while α and γ are molecular brightnesses of the free IDA and DNA-IDA complex, respectively.

Figure 39: Exemplary fit of equilibrium constant determination of (DNA-IDA)-IDA complex formation, at 36°C, in equilibrium state. Blue ▲ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM IDA solution in a titration experiment. The blue line corresponds to the fit of Equation (20). In the fit, C_{IDA} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the

complex formation reaction (treated as constant), K_2 is the equilibrium constant for the (DNA-IDA)-IDA complex formation reaction, and n denotes the binding site size.

Figure 40: Exemplary fit of equilibrium constant determination of DNA-EPI complex formation, at 36°C, in equilibrium state. Green ► represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM EPI solution in a titration experiment. The green line corresponds to the fit of Equation (15). In the fit, C_{EPI} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction, while α and γ are molecular brightnesses of the free EPI and DNA-EPI complex, respectively.

Figure 41: Exemplary fit of equilibrium constant determination of (DNA-EPI)-EPI complex formation, at 36°C, in equilibrium state. Green ► represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM EPI solution in a titration experiment. The green line corresponds to the fit of Equation (20). In the fit, C_{EPI} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction (treated as constant), K_2 is the equilibrium constant for the (DNA-EPI)-EPI complex formation reaction, and n denotes the binding site size.

Figure 42: The exemplary FLIM images of gels excited with a 485 nm laser. (A) FLIM image of gel containing 0.1% agarose and 6% gelatin. The bright, fluorescent spot of gelatin is observable. (B) FLIM image of gel containing 5% agarose. The gel does not give a fluorescence signal.

Figure 43: Exemplary FLIM images of phage λ DNA stained with YOYO-1 dye immobilised in 5% agarose gel. Various concentrations of DNA were used: (A) 25 pM, (B) 30 pM, (C) 35 pM and (D) 40 pM. Distances between centres of spherical DNA were measured. Mean values were equal: (A) 2.63 μm , (B) 2.48 μm , (C) 1.84 μm and (D) 1.75 μm .

Figure 44: Confocal image of HeLa cells stained with YO-PRO-1 dye. In the image, two cells are visible. Nucleoli, nuclear membrane and cytoplasm are stained with the dye. HeLa cells were incubated with 50 nM YO-PRO-1 dye for one hour.

Figure 45: Autocorrelation obtained from the fitting of FCS measurement of the cell lysate with YO-PRO-1 dye. Red and black (every tenth) points are experimental data (9 separate measurements), green line is the fitting of a one-component diffusion model, and violet line is the autocorrelation based on the averaged parameters.

Figure 46: Autocorrelation obtained from the fitting of FCS measurement of the cell lysate treated with RNase, with YO-PRO-1 dye. Red and black (every tenth) points are experimental data (9 separate measurements), green line is the fitting of a one-component diffusion model, and violet line is the autocorrelation based on the averaged parameters.

Figure 47: Autocorrelation obtained from the fitting of FCS measurement of the total pool of RNA, with YO-PRO-1 dye. Red and black (every tenth) points are experimental data, green line is the fitting of a two-component diffusion model with rotational diffusion of the bigger component, and violet line is the autocorrelation based on the averaged parameters.

Figure 48: Autocorrelation obtained from the fitting of FCS measurement of the tRNA, with YO-PRO-1 dye. The red and black (every tenth) points are experimental data, the green line is the fitting of a one-component diffusion model, and the violet line is the autocorrelation based on the averaged parameters.

List of tables

Table 1: Reported in literature experimental tRNA size.

Table 2: Reported in literature ribosome size.

Table 3: Reported in literature, small ribosomal subunit size.

Table 4: Reported in literature, large ribosomal subunit size.

Table 5: Reported in literature equilibrium constant values of the reaction between YOYO-1 dye and DNA.

Table 6: Reported in literature equilibrium constant values of the reaction between YO-PRO-1 dye and DNA.

Table 7: Reported in literature size of the binding site of YOYO-1 dye.

Table 8: Reported in literature size of the binding site of YO-PRO-1 dye.

Table 9: Collected photons per second for measured samples upon increasing laser power. The initial concentrations for the complex formation were 100 nM for DNA and 20 nM for the YO-PRO-1 dye reaction, and 20 nM for YOYO-1 dye in the reaction with 2 nM DNA. Reactions were performed at 25°C, in equilibrium state.

Table 10: Measured samples and collected photons per second of DNA-YO-PRO-1 complex, for all sets of titration experiments. The reaction was performed at 25°C. The initial concentration of YO-PRO-1 dye was 70 nM, and was kept constant. The DNA concentration was varied from 0.5 to 200 nM. " - "marks the incorrectly prepared sample, resulting in a non-physical value of the collected fluorescence intensity.

Table 11: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 25°C, in equilibrium state. The initial concentration of YOYO-1 dye was 20 nM. The dye concentration was kept constant. The DNA concentration ranges from 0.02 to 50 nM. " - "marks for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 12: Collected photons per second for measured samples for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YO-PRO-1 dye was 40 nM. The dye concentration was kept constant. The concentration of DNA varies between 1 and 100 nM. " - "marks for the incorrectly prepared sample that did not result in the non-physical value of collected fluorescence intensity in the measurement.

Table 13: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YOYO-1 dye was 20 nM. The dye concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 14: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YO-PRO-1 dye was 40 nM. The dye concentration was kept constant. The concentration of tRNA varies between 1 and 200 nM. " - "mark for the incorrectly prepared sample that results in a non-physical value in the measurement.

Table 15: Calculated molecular brightness (MB) (Eq. 4) for measured samples of DNA-YO-PRO-1 formed complex, for all sets of titration experiments, performed at 25°C, in equilibrium state. The initial concentration of YO-PRO-1 dye was 70 nM and was kept constant. The concentration of the DNA (69 bp) was varied from 0.5 to 200 nM. " - "marks the incorrectly prepared sample, resulting in a non-physical value of the collected fluorescence intensity.

Table 16: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 25°C, in equilibrium state. The initial concentration of YOYO-1 dye was 20 nM. The dye concentration was kept constant. The DNA concentration ranges from 0.02 to 50 nM. " - "marks for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 17: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium constant. The initial concentration of YO-PRO-1 dye was 40 nM, and was kept constant. The concentration of DNA varies between 1 and 100 nM. " - "marks for the incorrectly prepared sample that did not result in the proper value of collected fluorescence intensity in the measurement.

Table 18: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YOYO-1 dye was 20 nM. The dye concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 19: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YO-PRO-1 dye was 40 nM. The dye concentration was kept constant. The concentration of tRNA varies between 1 and 200 nM. " - "mark for the incorrectly prepared sample that results in a non-physical value in the measurement.

Table 20: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of doxorubicin was 100 nM. The drug concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 21: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial daunorubicin concentration was 100 nM. The drug concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 22: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of idarubicin was 100 nM. The drug concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 23: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial epirubicin concentration was 100 nM. The drug concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 24: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of doxorubicin was 100 nM. The drug concentration was kept constant. The concentration of DNA varies between 1 and 500 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 25: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial daunorubicin concentration was 100 nM. The drug concentration was kept constant. The concentration of DNA varies between 1 and 500 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 26: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of idarubicin was 100 nM. The drug concentration was kept constant. The concentration of DNA varies between 1 and 500 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 27: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial epirubicin concentration was 100 nM. The drug concentration was kept constant. The concentration of DNA varies between 1 and 500 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

Table 28: Compiled equilibrium constants and molecular brightnesses obtained from the analysed experimental data of the reactions between DNA and YO-PRO-1, and tRNA and YO-PRO-1, carried out at 25°C and 36°C, respectively.

Table 29: Compiled equilibrium constants and molecular brightnesses obtained from the analysed experimental data of the reactions between DNA and YOYO-1, carried out at 25°C and 36°C, separately. Equilibrium constants for the reactions of DNA are calculated based on base-pair concentration.

Table 30: Compiled equilibrium constants, fitted with averaged molecular brightnesses, of the reactions between YO-PRO-1 with DNA or tRNA, and DNA with YOYO-1, carried out at 25°C and 36°C, separately.

Table 31: Compiled equilibrium constants and molecular brightnesses obtained from the analysed experimental data of the reactions between DNA and anthracyclines, carried out at 36°C, separately. Equilibrium constants for the reactions of DNA are calculated based on base-pair concentration.

Table 32: Compiled equilibrium constants, fitted with averaged molecular brightnesses, of the reactions between DNA and anthracyclines, doxorubicin, daunorubicin, idarubicin and epirubicin, carried out at 36°C, separately.

Table 33: Collected mean radiuses of the focal volume, obtained from calibration in the 5% agarose and calculated from all consecutive experiments, with applied linear or circular polarisation of the laser beam, carried out at 25°C and 36°C, separately.

Table 34: Values of fluorescence intensities collected from the saturated with YO-PRO-1 dye phage λ DNA in buffer conditions, with applied linear or polarisation of the laser beam, at 25°C or 36°C, separately.

Table 35: Molecular brightnesses of DNA-YO-PRO-1 complex obtained from all consecutive experiments, with applied linear or circular polarisation of the laser beam, at 25°C or 36°C, separately.

Table 36: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with molecular brightnesses from consecutive experiments and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied linear polarisation of the laser beam, at 25°C.

Table 37: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with molecular brightnesses from consecutive experiments and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied circular polarisation of the laser beam, at 25°C.

Table 38: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with molecular brightnesses from consecutive experiments and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied linear polarisation of the laser beam, at 36°C.

Table 39: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with molecular brightnesses from consecutive experiments and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied circular polarisation of the laser beam, at 36°C.

Table 40: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with averaged molecular brightness and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied linear polarisation of the laser beam, at 25°C.

Table 41: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with averaged molecular brightness and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied circular polarisation of the laser beam, at 25°C.

Table 42: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with averaged molecular brightness and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied linear polarisation of the laser beam, at 36°C.

Table 43: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with averaged molecular brightness and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied circular polarisation of the laser beam, at 36°C.

Table 44: Fluorescence intensities of the nucleic acid samples without or with YO-PRO-1 dye, at 36°C.

1 Introduction

1.1 Nucleic acids

1.1.1 DNA

Nucleic Acids: Structure, Function, and Biological Significance

Deoxyribonucleic acid (DNA) is the fundamental polymer that carries the genetic instructions for development, function, and reproduction in all living organisms. Structurally, it consists of two phosphoryl-deoxyribose strands twisted into the iconic double helix. Each strand is built from repeating nucleotide units composed of a phosphate group, a deoxyribose sugar, and one of four nitrogenous bases: adenine (A), guanine (G), cytosine (C), or thymine (T) (Fig. 1). The strands are joined by hydrogen bonds between complementary bases—adenine with thymine, guanine with cytosine—which safeguard fidelity during replication (Fig. 2). Their antiparallel orientation ensures that each strand serves as a template for the synthesis of a new complementary strand, thereby preserving genomic integrity across generations [1].

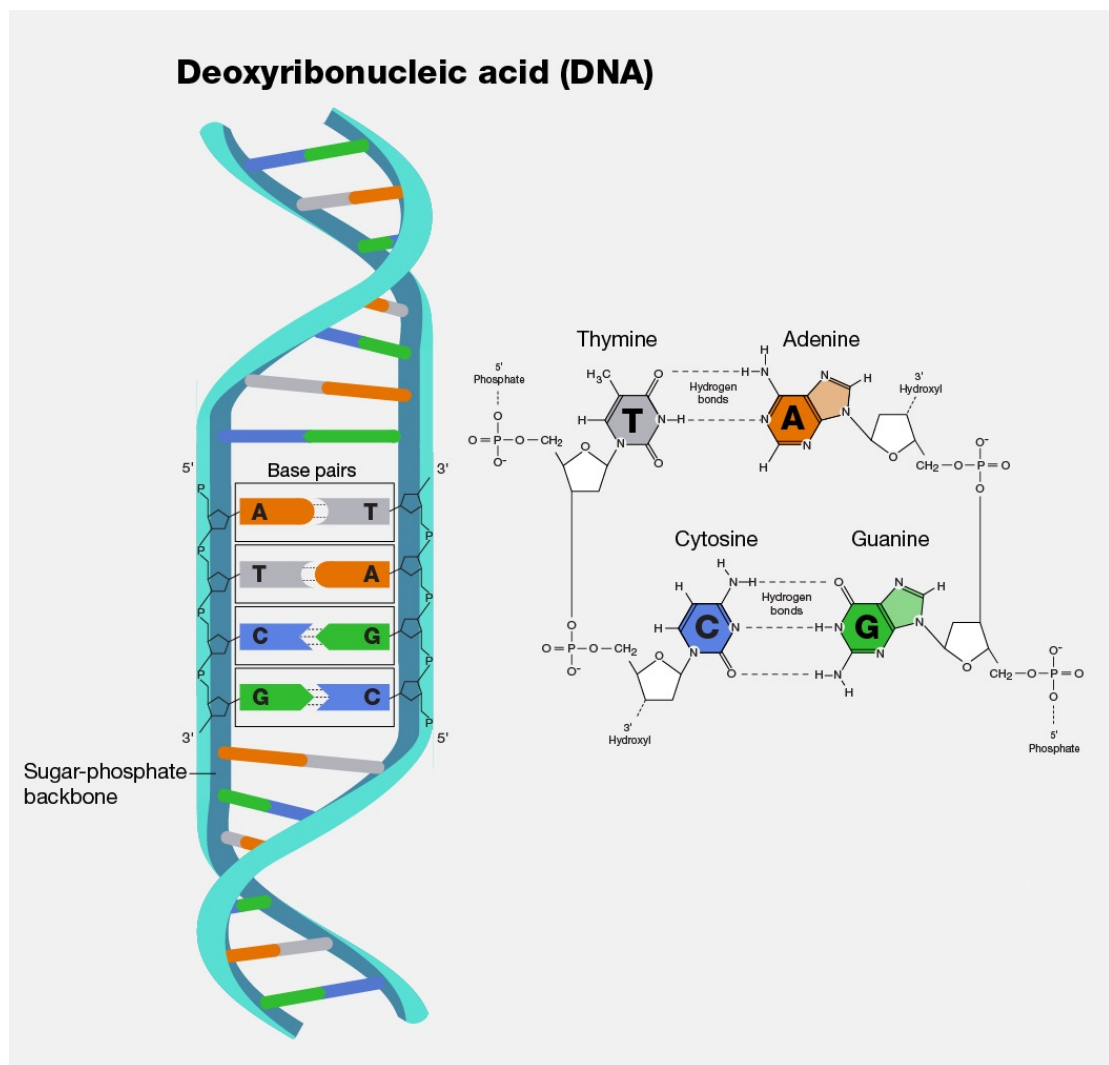


Fig. 1: Scheme of DNA helix structure and base pairing in the DNA structure [2].

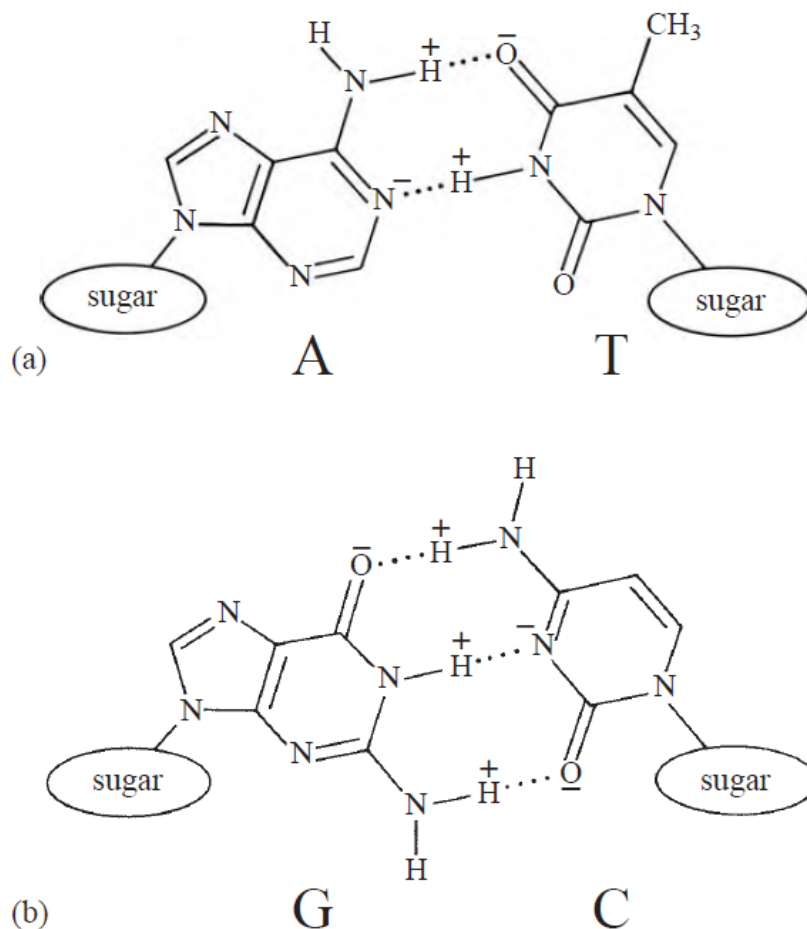


Fig. 2: Watson–Crick base pairs exhibit specific hydrogen bonding patterns: (a) adenine–thymine (A–T) pairs, and (b) guanine–cytosine (G–C) pairs. The key atoms involved in hydrogen bonding include nitrogen (N), oxygen (O), and hydrogen (H). Carbon atoms remain unlabelled for clarity. Hydrogen bonds stabilise the base pairing and are denoted by dotted lines. Partial electric charges, represented as + and -, highlight the polar nature of these bonds, typically amounting to about one-third of the charge of an electron or proton. [3].

The primary role of DNA is to store hereditary information. In humans, the genome contains about three billion base pairs, yet only 1.5% of this sequence encodes proteins. The remaining 98.5% is non-coding DNA, once dismissed as “junk” but now recognised as crucial for regulating gene expression. Regulatory elements, such as promoters and enhancers, provide binding sites for transcription factors, thereby controlling the activity of protein-coding genes [4].

A striking feature of non-coding DNA is its transcription into non-coding RNAs (ncRNAs), which account for up to 75% of the non-coding genome. Classical ncRNAs such as tRNAs and rRNAs are indispensable for protein synthesis, while newer classes—including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs)—function in post-transcriptional regulation, chromatin organisation, and diverse cellular processes. Their dysregulation has been linked to diseases ranging from cancer to neurodegenerative disorders, underscoring their biomedical significance [4].

Beyond its role as a carrier of genetic information, DNA also provides a structural scaffold for multiprotein complexes within the cell. Its water-soluble, locally

rigid double helix grants stability, while its inherent flexibility allows DNA-binding proteins to bend, wind, or unwind the helix. These dynamic interactions enable essential processes such as chromatin organisation, transcriptional regulation, and DNA replication [5].

From a biophysical perspective, DNA behaves as a semiflexible polymer with a persistence length of approximately 150 base pairs (50 nm). Over short distances, it maintains rigidity, while at larger scales it exhibits the flexibility characteristic of polymers. This property arises from base-pair stacking interactions and electrostatic repulsion between negatively charged phosphate groups [6]. The delicate balance between rigidity and flexibility is vital for genome organisation: it allows DNA to be compacted efficiently within the confined space of the nucleus while remaining sufficiently accessible for transcription, replication, and repair.

Physical Chemistry of DNA: Solvation, Non-Covalent Forces, and Ionic Modulation

Although nitrogenous bases are inherently water-insoluble, their covalent attachment to deoxyribose and phosphate produces water-soluble nucleotides, allowing DNA to function in the intracellular environment [3]. The hydrophobic effect drives bases into the duplex core while leaving the sugar-phosphate backbone solvent-exposed, minimising solvation penalties and ensuring structural integrity at neutral pH [3]. Backbone geometry is tightly constrained by 0.6 nm (Fig. 3) spacing between adjacent sugar or phosphate groups, while the rigid, planar bases (0.3 nm thick) leave 0.3 nm inter-base gaps along the helical axis (Fig. 4) [3].

Under physiological conditions, DNA adopts the B-form, with 10 bp per turn and a pitch of 3.4 nm. These parameters are critical for stability and biological function [7]. Stability derives from hydrogen bonding between complementary bases and π - π stacking, dispersion, and hydrophobic exclusion forces [8]. Base stacking contributes as much or more than hydrogen bonding, with stability highly sensitive to solvent polarity, ionic strength, and temperature [8]. In B-DNA, a 36° helical twist minimises Pauli repulsion between π -systems, while methyl repulsions fine-tune stacking [9]. GC-rich sequences gain extra robustness from diagonal and cross interactions, though solvation partly levels AT/GC differences [9].

Hydration further modulates DNA energetics. Water competes with inter-base hydrogen bonds, stabilising lone pairs and weakening/lengthening bonds, lowering melting temperatures and influencing base-pair breathing and recognition [8, 10]. In aqueous solution, neutral H-bonds contribute about $-1.5 \text{ kcal}\cdot\text{mol}^{-1}$ to stability; in duplex DNA their strength drops to $2\text{--}3 \text{ kcal}\cdot\text{mol}^{-1}$, far weaker than covalent bonds ($80\text{--}100 \text{ kcal}\cdot\text{mol}^{-1}$) [7, 11].

Electrostatics shape DNA architecture significantly. Monovalent cations like Na^+ and K^+ nonspecifically screen backbone charges, stabilising compact structures [12]. Divalent cations such as Mg^{2+} interact more specifically via coordinated waters and backbone oxygens, forming cross-strand H-bonds in the minor groove and engaging purine-rich surfaces in the major groove, modulating curvature and recognition [12]. Salt shields phosphate-phosphate repulsion, enhancing stacking and reducing fraying, with divalents being more effective than monovalents due to higher charge density and specific interactions [13].

Thus, DNA structure arises from a balanced network of non-covalent interactions, where hydrogen bonds provide complementarity and π -stacking offers

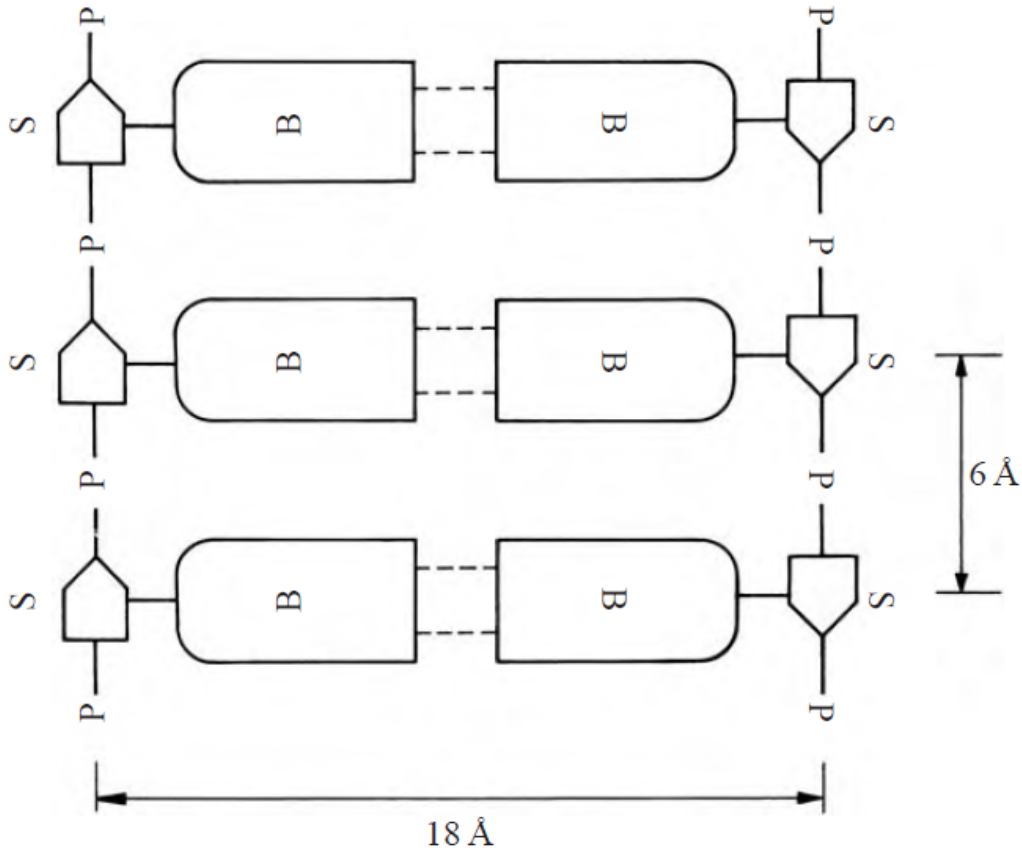


Fig. 3: A scheme of base pairs consecutive components with given distances [3].

stability, while solvation and counterions modulate these effects [3, 7, 8, 9, 10, 11, 12, 13, 14]. Divalent metal ions (M^{2+}) play a critical role by neutralising backbone charges, influencing DNA flexibility and structural transitions such as the A-to-B shift, which can be inhibited under low hydration [15]. Transition metals like Cu^{2+} , Co^{2+} , and Ni^{2+} can disrupt DNA structure and transcription, representing carcinogenic risks [15]. M^{2+} ions also interact with DNA-binding drugs. Small molecules like distamycin A mimic cationic coordination, while chemotherapy drugs like cisplatin crosslink DNA to block replication and induce apoptosis. Divalent ions can further stabilize drug binding, as seen with anthracyclines forming drug-metal-DNA assemblies with Cu^{2+} , affecting activity and cytotoxicity [15]. At high concentrations, cations like Cu^{2+} and Ca^{2+} can trigger helix collapse and alter DNA mechanics, with implications for chromatin and gene expression [15].

Altogether, divalent metal ions act as versatile regulators of DNA. They stabilise structures, control polymorphic transitions, and drive compaction while contributing to mutagenesis, carcinogenesis, and therapeutic targeting. Their ability to form ternary complexes with drugs underscores their pharmacological relevance, and their profound influence on DNA stability highlights their central role in genome regulation and disease.

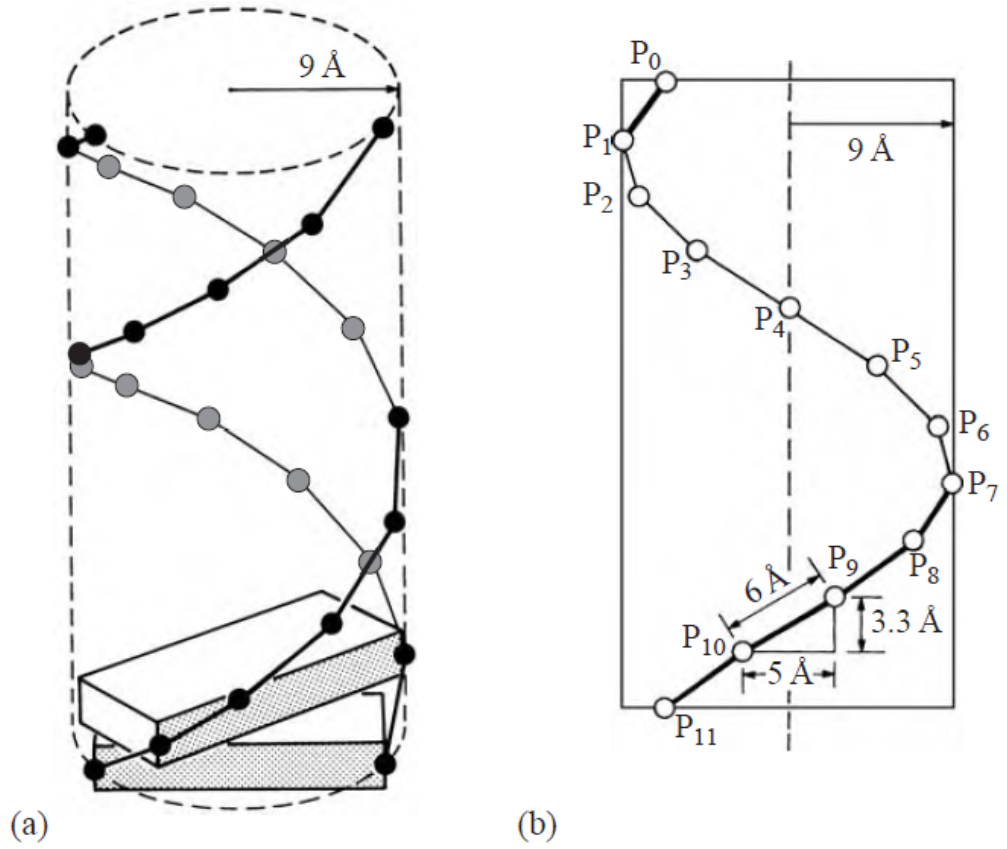


Fig. 4: The sugar-phosphate backbone of DNA can be visualised as helically wrapped chains around an imaginary central cylinder. Two distinct representations highlight this structure: (a) the sugar rings are depicted as shaded or filled circles, emphasising their position within the helix. The phosphate groups connecting the sugars are shown as thin lines, outlining the structural backbone. (b) The phosphate groups are represented as open circles, while the sugar rings are drawn as thin lines. This view shifts focus to the positioning of phosphates along the helix. The helical arrangement of the sugar-phosphate backbone is critical for DNA's stability and function [3].

1.1.2 RNA

Overview of RNA Diversity and Functional Classification

Ribonucleic acid (RNA) is a central molecule in gene expression, serving as both a carrier of genetic information and a regulator, catalyst, and structural component of cellular machinery. Although historically viewed primarily as a messenger between DNA and proteins, RNA is now recognised as a complex and multifunctional macromolecule with structural and regulatory capacities extending far beyond protein coding. Based on their cellular roles, RNAs can be broadly categorised into messenger RNAs (mRNAs) and noncoding RNAs (ncRNAs). [16]

Messenger RNA (mRNA) directs protein synthesis by transporting genetic information from DNA to ribosomes, where it is translated into proteins. Ribosomal RNA (rRNA) forms the catalytic core of ribosomes and facilitates peptide bond formation, whereas transfer RNA (tRNA) decodes the mRNA sequence into corresponding amino acids during translation. [16, 17] Together, rRNA and tRNA constitute approximately 90% of total cellular RNA, while mRNA accounts for only 1–5%, underscoring the dominant role of ncRNAs in cellular RNA pools. [17]

Despite the small proportion of protein-coding sequences, the majority of the human genome is actively transcribed into ncRNAs. These include rRNAs, tRNAs, microRNAs (miRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), riboswitches, and many other species. [16, 18, 19] Increasing evidence suggests that organismal complexity correlates more strongly with ncRNA repertoire than with genome size itself, highlighting the pivotal regulatory functions of RNA in cellular systems. [20, 21]

RNAs operate across all levels of cellular regulation—modulating transcription, translation, chromatin organisation, post-transcriptional processing, and intracellular signalling—and are deeply implicated in diverse disease states. [20, 22] These discoveries have expanded the conceptual boundaries of RNA biology and positioned RNA as both a key regulatory element and an attractive therapeutic target.

Hierarchical Principles of RNA Structure

Primary Structure and Chemical Fundamentals

RNA structure is organised hierarchically into primary, secondary, tertiary, and quaternary levels. The primary structure consists of a linear sequence of ribonucleotides joined by phosphodiester bonds. Each nucleotide contains a ribose sugar linked via a glycosidic bond to one of four nucleobases—adenine (A), guanine (G), cytosine (C), or uracil (U) (Fig. 5). [23]

A defining chemical feature distinguishing RNA from DNA is the presence of a 2'-hydroxyl group on the ribose sugar. This hydroxyl group not only increases backbone reactivity and conformational flexibility but also promotes A-form helical geometry. Electrostatic repulsion between phosphate groups is mitigated by metal ions such as Mg^{2+} , which are essential for RNA folding into compact secondary and tertiary structures. [23]

Two fundamental interactions dominate RNA structural formation: hydrogen bonding (base pairing) and π - π stacking interactions between aromatic nucleobases. Together, these interactions stabilise RNA helices and provide the foundation for higher-order organisation. [24]

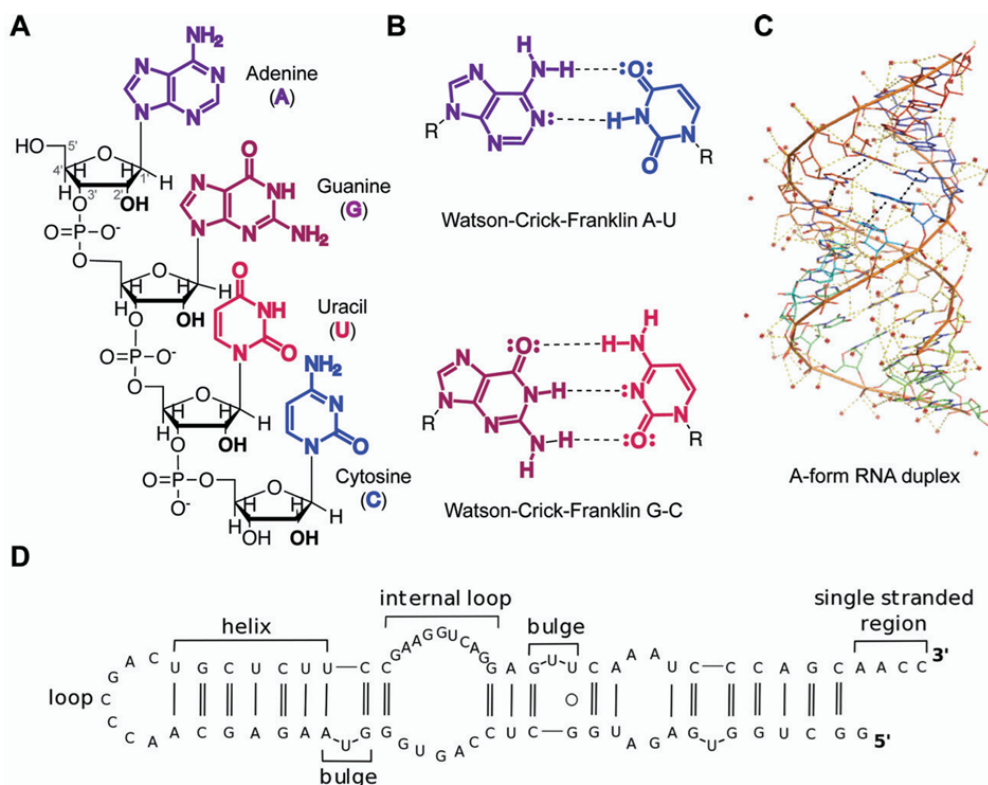


Fig. 5: Overview of RNA Primary and Secondary Structure and Base Pairing Patterns. (A) The nucleotide building blocks of RNA primary structure consist of a ribose sugar, which contains a 2' hydroxyl group unique to RNA compared to DNA; phosphodiester groups that connect the 5' and 3' hydroxyl groups of ribose sugars; and the nucleobases adenine, guanine, uracil, and cytosine, which define the RNA sequence. (B) Canonical Watson–Crick–Franklin hydrogen bonding patterns, with dashed lines indicating hydrogen bonds. (C) An A-form RNA duplex secondary structure. Red dots denote metal ions, yellow dashes highlight electrostatic interactions such as hydrogen bonds, and black dashes indicate stacking interactions. (D) Representative canonical RNA secondary structures from the 5' end of the long non-coding RNA (lncRNA) B2-SINE. Single and double solid lines represent A–U and G–C Watson–Crick–Franklin base pairs, respectively, while circles denote wobble base pairs. Figure adapted from [23]

Secondary Structure: Helices, Loops, and Dynamic Topologies

RNA is typically single-stranded but frequently folds back on itself to form intramolecular hydrogen-bonded helices. The canonical base pairs—Watson–Crick A–U and G–C—form stable A-form duplexes, supported by cooperative base stacking. [23] Secondary structural elements include stems, hairpins, internal loops, bulges, multi-helix junctions, and pseudoknots, all of which contribute to RNA's architectural diversity. [25, 26]

Beyond canonical Watson–Crick interactions, RNA secondary structures often incorporate non-canonical pairs, including G–U wobble pairs, Hoogsteen interactions, and sheared purine–purine pairs. These motifs expand RNA's structural repertoire, modulate local geometry, and often act as recognition elements for proteins and small molecules. [23, 24, 27]

The dynamic nature of secondary structure is central to RNA function. RNA molecules continually sample alternative base-pairing configurations, enabling them to respond rapidly to cellular cues and adopt the structures necessary for regulatory activity. [28, 29]

Tertiary Structure: Long-Range Interactions and Folding Principles

Tertiary structure arises when secondary structure elements interact over long distances. Stabilising interactions include coaxial stacking, base triples, pseudoknots, kissing loops, A-minor motifs, and metal ion coordination. [23, 30]

The folding of RNA is primarily driven by the optimisation of base stacking, analogous in some respects to the protein-folding hydrophobic effect. [23] Complex RNA tertiary structures, such as those in ribozymes, tRNAs, rRNAs, and self-splicing introns, are highly organised three-dimensional architectures essential for catalysis and molecular recognition. [26, 30, 31]

Specialised tertiary motifs include G-quadruplexes, assembled from guanine-rich sequences that form stacked G-quartets stabilised by monovalent cations. These structures play key regulatory roles and have been implicated in human disease. [23]

Quaternary Structure and RNA-Protein Assemblies

RNA frequently associates with proteins, other RNAs, and small molecules to form ribonucleoprotein (RNP) complexes, which constitute quaternary structures. Protein-RNA binding relies on hydrogen bonding, aromatic stacking, and electrostatic interactions with the negatively charged backbone. [23]

RNA-protein interactions are essential for splicing (snRNPs), translation (ribosomes), RNA transport, RNA turnover, and numerous regulatory processes. [27, 30] These complexes serve as central hubs of gene expression and are increasingly viewed as promising targets for therapeutic intervention. [20, 23]

Determinants of RNA Folding and Structural Dynamics

Physicochemical Influences on RNA Conformation

Osmolytes can promote RNA compaction by driving dehydration, while the crowded intracellular milieu constrains conformational freedom, favouring compact states. [25, 30]

Molecular Modulators and Enzymatic Remodeling

RNA interacts extensively with proteins, metabolites, and other RNAs, all of which modulate its folding pathways. Riboswitches undergo ligand-induced structural rearrangements, RNA-binding proteins guide folding or stabilise specific conformations, and helicases remodel structures using ATP. [25, 31]

Chemical modifications, such as methylation or pseudouridylation, alter local stability, protein affinity, and structural preferences, introducing an additional layer of regulation. [32]

Non-equilibrium Dynamics and Conformational Ensembles

Unlike many theoretical models, cellular RNA seldom occupies a single minimum-energy structure. Instead, RNAs exist within dynamic ensembles of conformations, often including kinetically trapped states that are nonetheless functional. [25, 28, 29]

Sequence composition enables alternative pairing possibilities, and noncanonical interactions broaden the range of accessible structures. Tertiary contacts—such as long-range loop-loop interactions, bulges, and junctions—further stabilise alternative conformers. [23, 24, 28]

Chemical probing approaches, including large-scale *in vivo* reactivity mapping,

have been instrumental in resolving these structural ensembles, though interpretation requires careful consideration of potential biases. [33]

Functional RNAs in Gene Expression and Regulation

Splicing, Catalysis, and Regulatory Architecture

Many fundamental cellular processes — including translation and splicing — are mediated by structured RNAs. Ribosomes, spliceosomes, and self-splicing introns exemplify ribonucleoprotein complexes in which RNA adopts catalytic roles. [30, 31]

Alternative splicing, which affects nearly 95% of human protein-coding genes, is deeply influenced by RNA structural elements that undergo tissue- and time-specific rearrangement. These structures modulate splice-site choice and are shaped by both *cis*- and *trans*-acting factors. [34]

Small Regulatory RNAs: siRNAs, miRNAs, and RNAi

Small RNAs, ranging in length from 21 to 24 nucleotides, mediate RNA interference and post-transcriptional gene silencing. siRNAs derive from long dsRNAs processed by Dicer, then guide Argonaute proteins to complementary targets for cleavage. [18, 35]

miRNAs originate from hairpin precursors (pri-miRNAs), undergo Drosha and Dicer processing, and direct RISC-mediated translation repression. Intronic miRNAs coordinate regulatory networks by linking transcription and post-transcriptional control. [18]

piRNAs associate with Piwi proteins and suppress transposons through epigenetic and post-transcriptional silencing, safeguarding genome stability. [18] Small activating RNAs (saRNAs) represent an additional class that promotes transcription activation rather than repression. [18]

Long Noncoding RNAs, Circular RNAs, and RNA Networks

LncRNAs (>200 nt) regulate chromatin architecture, transcription, nuclear organisation, mRNA stability, and nuclear body assembly. [18, 36] Despite extensive annotation, their localisation and cell-to-cell variability remain incompletely characterised. [37]

Circular RNAs (circRNAs), generated through backsplicing, form covalently closed loops enriched in specific tissues and developmental stages. They may act as miRNA sponges, regulators of protein function, or templates for translation. [18, 38, 39]

Together, these ncRNAs form interconnected regulatory networks that control gene expression at multiple levels. [19]

RNA in Disease, Molecular Recognition, and Therapeutic Innovation

Structural Ensembles and Disease Mechanisms

RNA structural heterogeneity contributes to both regulatory function and disease. Rare structural states can misregulate gene expression or generate pathogenic interactions and are now considered promising therapeutic targets. [22, 29]

A deep understanding of these ensembles is essential for deciphering RNA-mediated control of gene expression and for developing structure-targeted therapies. [40]

RNA as a Therapeutic Target and Tool

RNA is increasingly recognised as an attractive therapeutic target and modality. Unlike protein-based drugs, RNA therapeutics are relatively simple to design, cost-effective to produce, and uniquely capable of modulating pathways previously considered undruggable. [21, 41]

RNA therapeutics include: (1) mRNAs — encoding therapeutic proteins or vaccine antigens, [41, 42, 43] (2) Antisense oligonucleotides (ASOs) — directing degradation or splice modulation of target RNAs, [18, 42, 43] (3) siRNAs and miRNAs — suppressing mRNA translation or expression, [18, 43] (4) Aptamers — structured RNAs that bind proteins or small molecules with high specificity [44] and (5) Guide RNAs — directing CRISPR-based genome editing. [43, 45]

Recent therapeutic successes include mRNA vaccines, antisense therapies for genetic disorders, and siRNA drugs for metabolic and neurological diseases. [42, 43]

RNA Delivery, Immune Response, and Clinical Application

Early limitations in RNA therapeutics centred around rapid degradation, inefficient delivery through the hydrophobic membrane, and innate immune activation by foreign RNA. Advances in chemical stabilisation, lipid nanoparticle delivery, and sequence optimisation have overcome many of these barriers. [41, 43]

In oncology, the *ex vivo* or *in vivo* delivery of tumour-antigen mRNAs to dendritic cells triggers robust immune responses, demonstrating the versatility of RNA-based therapeutic design. [43]

Together, these developments position RNA therapeutics as a transformative class of medicines with broad applicability across genetic, infectious, metabolic, and oncological diseases. [41, 43, 44]

The study of drug- and dye-RNA interactions is essential for the development and optimisation of RNA-targeted therapeutic strategies. Such interactions can provide information about RNA concentration, localisation, structural state, and binding behaviour, thereby supporting both mechanistic studies and the design of diagnostic or therapeutic tools. Fluorescent dyes, in particular, enable the visualisation and quantification of RNA in complex biological environments, while small-molecule drugs can selectively modulate RNA structure or function. Understanding these interactions is therefore important not only for analytical detection but also for the rational development of targeted RNA-based therapies.

1.1.3 Messenger RNA

Messenger RNA: structure, regulation, and functional significance

Fundamental organisation and biological role of mRNA

Messenger RNA (mRNA) is a transcribed copy of a defined DNA segment, typically several thousand nucleotides in length. Its central function is to convey genetic information to the translational machinery, which uses it as a template for protein synthesis. The coding region of the transcript determines the amino acid sequence of the synthesised protein, whereas the 5' and 3' untranslated regions (UTRs) contribute essential regulatory functions without being translated themselves. [23, 26] mRNA therefore represents the transient molecular link between genomic DNA and cellular protein production. [41]

Before translation can occur, pre-mRNA undergoes extensive maturation, including intron removal through splicing and processing of both its 5' and 3' termini. The resulting mature mRNA contains numerous cis-regulatory elements that determine its trafficking, stability, degradation, and translational potential, integrating multiple layers of post-transcriptional gene regulation. [46]

Primary sequence, structural organisation, and the multilayered information encoded in mRNA

Although the primary role of mRNA is to encode proteins, the molecule is far more than a linear array of codons. It is a structurally complex macromolecule whose functional properties arise from both its primary nucleotide sequence and its propensity to form secondary and tertiary structures. [47] Messenger RNAs carry information at multiple levels: the encoded amino acid sequence, the kinetics of translation, and the structural features that influence their own stability and regulatory behaviour. [48]

Single-stranded mRNAs spontaneously fold into secondary structures via intramolecular base pairing, and several hypotheses suggest that these structures are intimately linked to the underlying nucleotide sequence and even to the amino acid sequence specified by the coding region. [49] Transcriptome-wide structural analyses have revealed global correlations between the structural content of specific mRNA regions and the abundance of their encoded proteins. [48]

The UTRs of mRNAs harbour numerous regulatory structural elements — stem-loops, long-range interactions, G-quadruplexes, R-loops, and pseudoknots — that substantially affect mRNA synthesis, splicing, export, stability, and translation. [50] These motifs provide binding platforms for RNA-binding proteins and other trans-acting regulators, thereby shaping the localisation, stability, and translational competence of individual transcripts. [46, 51]

mRNA structure as a regulator of translation

Initiation of translation and structural determinants

Translation efficiency depends on the success of initiation and elongation on the coding sequence, as well as on the functional half-life of the mRNA — all features encoded within its primary sequence. [48] Among the stages of translation, initiation is the principal rate-limiting step. Translation efficiency varies widely between cell types and among transcripts, often by several orders of magnitude, and much of this variability is encoded in mRNA structural features. [52]

Secondary structures that overlap the start codon must be unwound before

translation begins, imposing an energetic cost that reduces the ribosome’s access to the initiation site. [52] The stability and positioning of these structures, therefore, constitute key determinants of translational control.

Elongation, co-translational folding, and ribosomal kinetics

Although initiation has traditionally been regarded as the dominant regulatory step, studies over the past two decades demonstrate that elongation is equally essential. It shapes the rate of protein production and coordinates the co-translational folding of emerging polypeptides. [47] Secondary structure elements within mRNAs — notably helices, loops, and more complex structural motifs — can impede ribosomal progression, reducing translational throughput. [47]

Thus, mRNA encodes both the primary sequence of a protein and the structural signals that modulate translation dynamics, including elongation rate, ribosome pausing, and nascent chain folding trajectories. [47, 48] Despite extensive insights from individual transcripts, systematic analyses linking protein abundance directly to mRNA structural features remain scarce. [53]

Because RNA structure strongly influences translational efficiency, any ligand-induced structural perturbation may potentially alter protein production. Intercalation, by inserting between stacked bases, can modify local RNA conformation, stability, and accessibility, raising the question of whether such binding events interfere with translation. Therefore, studies of intercalating dye–RNA interactions are relevant not only for characterising binding mechanisms, but also for understanding whether structural changes induced by intercalation can affect RNA function. In this context, the present research may help clarify how intercalation affects RNA behaviour and its translational potential.

Post-transcriptional regulation, disease relevance, and mRNA fate

Post-transcriptional regulation involves multiple layers, including splicing, nuclear export, localisation, stability, and translation. The UTRs of mRNAs, together with the diverse structural elements they contain, act as integrators of signals from RNA-binding proteins, noncoding RNAs, and other regulatory factors. [23, 26, 46]

These regulatory systems are frequently disrupted in disease. In cancer, mutations in splicing factor genes and widespread shortening of 3’ UTRs alter the regulatory landscape of mRNAs. Additionally, accumulating evidence indicates that other RNA species, such as circular RNAs, contribute significantly to tumorigenesis. [54]

Principles and applications of mRNA-based medicine

The biochemical properties of mRNA — its transience, programmability, and lack of genomic integration — make it a promising therapeutic platform. Compared with recombinant proteins or traditional small-molecule drugs, mRNA therapeutics are easier and more cost-effective to produce, and their sequences can be rapidly designed and customised. [41]

Currently, mRNA is applied in three major therapeutic strategies: (1) replacement therapy, in which mRNAs encoding defective or absent proteins are delivered to restore function; (2) mRNA vaccines, which induce immune responses by expressing antigenic proteins; and (3) cell-based therapies, where mRNAs are introduced into cells *ex vivo* to modulate their phenotype before reinfusion. [41]

In all these contexts, the same regulatory principles that govern endogenous

mRNA behaviour — including UTR architecture, structural dynamics, and stability — are essential considerations for designing safe and effective therapeutics.

1.1.4 Transfer RNA

General role, genomic organisation and abundance

Transfer RNAs (tRNAs) are small noncoding RNAs (Table 1) that act as adaptor molecules, translating the triplet nucleic acid code of messenger RNA (mRNA) into the amino acid sequence of proteins. [1, 55, 56] By recognising codons via their anticodons and delivering the corresponding amino acids to the ribosome, tRNAs bridge the informational level of nucleic acids with the functional level of proteins and are therefore central to genetically encoded protein synthesis. [26, 57, 58, 59]

In human cells, tRNAs are among the most abundant RNA species, accounting for about 10% to 15% of total RNA and reaching tens of millions of molecules per cell. [60, 61, 62, 63] The classical experimental estimate of the number of human tRNA genes is 1,310, and these genes are dispersed throughout the genome in a non-random manner. While local clusters are observed at small scales, genome-wide analyses reveal substantial clustering, with more than 25% of tRNA genes (140 loci) residing in a ~ 4 Mb region on chromosome 6. [1, 64] Based on their aminoacylation identity, tRNAs are subdivided into 20 accepting groups (alloacceptors), each comprising several isoacceptor tRNAs that translate synonymous codons with the same amino acid. [55, 56, 65] Together, these features ensure that every amino acid encoded in the genetic code is matched by one or more cognate tRNA species.

Table 1: Reported in literature experimental tRNA size.

R [nm]	Reference
2.67 - 2.80 nm	[66]
2 nm	[67]
2.6 nm	[68]

Molecular architecture of tRNA

Primary and secondary structure

Canonical tRNAs are short biopolymers of approximately 70–90 nucleotides, composed of the four standard ribonucleotides (A, C, G and U) linked by 3'–5' phosphodiester bonds. [26, 69, 70] Their internal base pairing gives rise to a characteristic cloverleaf secondary structure consisting of the acceptor stem, D-arm, anticodon arm, variable loop and T-arm (Fig. 6). [26, 55, 56, 59, 64, 71] The acceptor stem joins the 5' and 3' ends of the molecule; the D-loop, rich in dihydrouridine, contributes to stabilisation of the tertiary fold; the anticodon loop carries the anticodon triplet that recognises mRNA codons; the variable loop exhibits the greatest diversity in size and sequence; and the T-loop contains conserved sequence and structural motifs required for ribosomal interactions. [26, 55, 56, 71]

The acceptor and T-arms typically contain 7 and 5 base pairs, respectively, forming the canonical 7/5 configuration. [71] Occasional mismatches in the acceptor stem generally do not disrupt the overall helical architecture. At the 3' end of the acceptor stem, there is the discriminator base and the conserved CCA sequence, whose terminal adenosine is esterified to the cognate amino acid during aminoacylation. [26, 70, 71] The anticodon loop typically comprises 7 nucleotides,

with the central three forming the anticodon. These nucleotides engage in Watson–Crick or wobble base pairing with complementary codons on mRNA, allowing both high fidelity and flexibility in decoding. [26, 55, 56, 71]

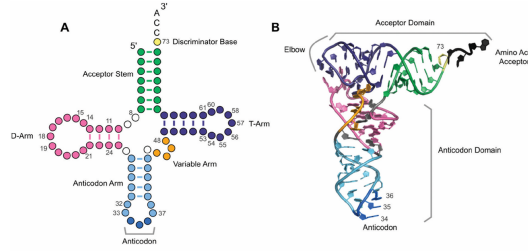


Fig. 6: tRNA structure. (A) Two-dimensional cloverleaf representation of tRNA. From the 5' to the 3' end, the structural elements include the acceptor stem (green), D-arm (pink), anticodon arm (blue), which contains the anticodon (highlighted in darker blue), the variable arm (orange), and the T-arm (purple). The discriminator base (position 73, shown in yellow) lies unpaired at the 3' end, immediately before the terminal CCA residues. Aminoacyl-tRNA synthetases catalyse the attachment of an amino acid to the terminal 3' adenosine. Numbered positions indicate conserved bases. (B) Three-dimensional structure of yeast tRNA^{Phe}. The same colour scheme as in panel (A) is used to highlight the structural regions. Intramolecular interactions between the D-arm (pink) and the T-arm (purple) form the elbow region, a critical feature that facilitates proper tRNA folding into its characteristic L-shaped structure. [56]

Tertiary structure, conserved core and identity elements

In three dimensions, the cloverleaf folds into a compact L-shaped tertiary structure. [55, 56, 69, 71, 72] This conformation is stabilised by extensive base stacking and tertiary interactions, particularly between the D- and T-loops. One arm of the L is formed by coaxial stacking of the acceptor stem with the T-stem, whereas the other arises from stacking of the D-stem with the anticodon stem. [55, 56, 59, 69, 71] The resulting architecture comprises two functional domains that are arranged approximately perpendicular to each other: an aminoacylation domain (acceptor and T arms) and an anticodon domain (anticodon and D arms). [72] These domains are functionally distinct and provide the positional framework for bridging codon recognition on mRNA with amino acid delivery during translation. [69, 72]

Despite substantial diversity in length (about 65–88 nucleotides), sequence (with an average pairwise identity of $\sim 50\%$) and biological context (e.g. association with different proteins), pivot-based multiple alignment of 27 representative tRNA structures has identified a conserved three-dimensional core composed of 14 nucleotides. [72] These conserved positions are predominantly located within the aminoacylation domain, accounting for approximately 40% of its composition, whereas the anticodon domain is markedly more variable. [72] A parallel pattern of conservation is observed in aminoacyl-tRNA synthetases (aaRSs): regions interacting with the aminoacylation domain are highly conserved, while those engaging the anticodon domain are more divergent. [72] These observations support an evolutionary model in which the aminoacylation domain and its synthetase recognition surface arose early, with the anticodon domain emerging later to link amino acid recognition to the triplet genetic code. [72]

Biogenesis, maturation and post-transcriptional modification

tRNA processing

tRNAs are produced as precursor transcripts (pre-tRNAs) that undergo a series of maturation steps before becoming fully functional. [60, 73, 74] These

steps include endonucleolytic and exonucleolytic removal of 5' leader and 3' trailer sequences, as well as splicing reactions to excise introns present in several tRNA precursors. [62, 73, 74]

Diversity, localisation and roles of tRNA modifications

Following maturation, tRNAs undergo extensive post-transcriptional chemical modification. More than 170 RNA modifications have been described, and approximately half of these occur in tRNAs, making them the most heavily and chemically diverse modified RNAs. [32, 64, 65, 70, 75] Eukaryotic tRNAs contain, on average, about 13 modifications per molecule, including base or ribose methylation, pseudouridylation, acetylation, deamination, thiolation and more complex ring structures. [32, 60, 65, 70, 75]

These modifications can be broadly categorised into two groups. The first comprises the majority of modified bases, introduced by single enzymatic reactions, distributed throughout the tRNA but particularly dense in the tRNA core, where tertiary interactions between the D- and T-arms stabilise the overall three-dimensional fold. [74, 75] The second group consists of more complex hypermodifications generated by the sequential activity of multiple enzymes, localised predominantly in the anticodon loop, where they maintain its structure and ensure accurate and efficient translation. [64, 70, 74]

Canonical role in translation and decoding

Aminoacylation and substrate function in the ribosome

The primary function of tRNA is to act as an adaptor that converts genetic information into a protein sequence. [26, 55, 57, 59] Each tRNA is charged with its cognate amino acid by a specific aaRS, yielding an aminoacyl-tRNA (aa-tRNA) that serves as the direct substrate for peptide bond formation at the ribosome. [26, 59, 70, 71] During translation, aa-tRNAs interact with mRNA codons, ribosomal RNA and proteins, and elongation factors, enabling the stepwise incorporation of amino acids into a growing polypeptide chain according to the genetic code. [57, 58]

Isoacceptor tRNAs, carrying the same amino acid but bearing distinct anticodons, facilitate decoding of synonymous codons and permit fine-tuning of translation efficiency across different genes. [55, 65] Accurate translation requires both correct codon recognition and faithful aminoacylation; errors in either process lead to mistranslation. [57, 64]

Mistranslation, proteostasis and adaptive decoding

Given the complexity of interactions among tRNAs, aaRSs, ribosomes, elongation factors and modifying enzymes, translational errors are inevitable. The frequency of mistranslation is approximately 1 error per 10^3 – 10^4 codons under standard conditions. [57] Such errors can compromise proteostasis and threaten cell viability through the accumulation of misfolded or dysfunctional proteins. [57, 63] Nonetheless, emerging evidence indicates that controlled mistranslation can, in specific contexts, be exploited as a survival mechanism, allowing cells to adapt to environmental challenges. [57]

tRNA abundance, aminoacylation levels and modification patterns collectively shape decoding kinetics and accuracy in a cell-type- and environment-dependent manner. [63, 64, 65] How these parameters are integrated to optimise translational

efficiency while maintaining acceptable error rates remains an important open question. [57, 63]

Noncanonical functions and regulatory roles of tRNA

tRNA-derived fragments and RNA-mediated regulation

Beyond their canonical role in translation, tRNAs give rise to tRNA-derived fragments (tRFs), small RNAs that contribute to a wide spectrum of regulatory processes. [59, 60, 63, 69, 73] tRFs have been implicated in the regulation of protein translation, Argonaute-dependent gene silencing, cellular stress responses and epigenetic inheritance in germ-line cells. [59, 60, 63, 75] Their abundance across species and conditions underscores their importance as additional layers of RNA-based regulation. [63, 73, 76]

Metabolic, signalling and genomic functions

tRNA^{Arg} mediates amino-terminal arginylation, a degradation signal in protein quality control pathways. [55, 62] Mitochondrially encoded tRNAs can interfere with cytochrome c-mediated apoptosis, promoting cell survival, and nuclear tRNA genes function as chromatin insulators segregating transcriptionally active and silent domains. [62] Furthermore, specific cellular tRNAs or their fragments act as primers for viral reverse transcription, coupling tRNA biology to retroviral replication. [55, 59, 62]

tRNAs also interact with numerous proteins that are not directly involved in translation, suggesting that they help coordinate protein synthesis with other cellular processes, including energy metabolism and amino acid biosynthesis. [62, 63, 73]

tRNA dynamics in stress responses

Cells are continuously exposed to environmental and physiological stressors, such as nutrient deprivation, heat shock, viral infection and oxidative stress. [62, 73] To cope with these challenges, they reprogramme gene expression, including the activity of the translational apparatus and the behaviour of tRNAs. Under various stress conditions, cytoplasmic tRNAs can rapidly relocate to the nucleus, reducing their availability in the cytoplasm and directly limiting global protein synthesis. [73] Concomitantly, stress-induced changes in tRNA abundance, charging levels and modification patterns contribute to selective translation of specific mRNA subsets, thereby supporting cellular adaptation. [57, 63, 64, 65]

tRNA stability, disease associations and clinical relevance

Structural robustness and RNA stability

tRNA molecules are among the most stable RNAs in the cell. In contrast to mRNAs, which often have half-lives measured in minutes to hours, tRNA half-lives typically span several days. [63, 76] This extreme stability is conferred by their compact, highly folded secondary and tertiary structures, dense networks of base stacking and tertiary contacts, and extensive post-transcriptional modifications. [58, 69, 74, 76] As a result, tRNAs, together with rRNAs, are among the most robust RNA species in the cellular environment. [76]

tRNA modifications, abundance and human disease

Perturbations in tRNA modifications and homeostasis are linked to a wide variety of human diseases. [62, 63, 64, 65, 75] Mutations in tRNA modification enzymes are associated with neurological dysfunction, mitochondrial disorders and

developmental syndromes, while DNA amplifications and overexpression of these enzymes are common in many cancers. [63, 64, 65, 75] Human mitochondrial tRNA mutations are a major cause of diseases such as MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonic epilepsy with ragged-red fibres), underscoring the requirement for correct tRNA structure, modification and aminoacylation in organellar function. [63]

Aberrant tRNA overexpression, dysregulated tRNA pools, and altered modification patterns contribute to oncogenesis and tumour progression by reshaping translational programmes and promoting abnormal protein synthesis. [62, 63, 64] Mutations in tRNAs or factors involved in tRNA biogenesis often display striking tissue-specific disease manifestations, suggesting that cell-type-specific contexts modulate the penetrance and severity of tRNA-related pathologies. [62, 63]

Collectively, these insights establish tRNAs as indispensable components of the translational machinery, versatile regulatory molecules, and key determinants of cellular homeostasis and human disease. [57, 63, 73, 76]

1.1.5 Ribosomal RNA

Architecture and composition of the ribosome

Ribosomes are large ribonucleoprotein machines that decode the genetic information embedded in mRNAs into polypeptides and thus constitute the core protein synthesis apparatus of all cells. [26, 77] They are present in very high copy numbers: an average human cell contains approximately 10^6 – 10^7 ribosomes, each with a size on the order of 25–30 nm (Table 2). [26, 78, 79, 80] Because ribosomes must be duplicated at each cell division and continually replaced as they turn over, ribosome production represents one of the most energetically demanding processes in the cell. [79]

Across all domains of life, ribosomes are organised into two unequal subunits: a small subunit (SSU), dedicated largely to mRNA decoding, and a large subunit (LSU), which catalyses peptide bond formation. [26, 77, 81] In mammalian cells, the cytosolic 80S ribosome consists of a 40S SSU and a 60S LSU (Tables 3 and 4). [82, 83, 84, 85] The 40S subunit contains 18S rRNA in complex with 33 ribosomal proteins (RPs), whereas the 60S subunit contains three rRNAs (28S, 5.8S and 5S) and 43 RPs, for a total of four non-coding RNAs and ~ 80 RPs per ribosome. [79, 83, 84, 85] The human 80S ribosome has an approximate mass of 4.3 MDa and represents a paradigmatic example of evolutionary conservation: despite profound differences in translation regulation, particularly at the initiation step, 33 of the 80 human ribosomal proteins have conserved homologues in *Escherichia coli*, which diverged from humans 3–4 billion years ago. [79] Overall, ribosomal RNA (rRNA) accounts for about 80% of total RNA in mammalian cells and 80–90% of total RNA mass, underscoring its quantitative dominance in the transcriptome. [79, 83]

Table 2: Reported in literature ribosome size.

R [nm]	Reference
24 nm $\times$ 30 nm	[?]
20-30 nm	[?]

Table 3: Reported in literature, small ribosomal subunit size.

R [nm]	Reference
23 nm $\times$ 14 nm	[?]
16.5 - 17 nm	[?]
27 $\times$ 18 $\times$ 16 nm	[?]
26 $\times$ 17 $\times$ 13 nm	[?]

Table 4: Reported in literature, large ribosomal subunit size.

R [nm]	Reference
20 nm $\times$ 24 nm	[?]

rRNA as catalytic and structural core of the translational machinery

Although ribosomes are ribonucleoprotein assemblies, rRNA constitutes the bulk of their mass and performs most of their essential functions. [79, 81, 83, 86] High-resolution X-ray crystallography, combined with biochemical and genetic evidence, indicates that peptide bond formation is catalysed by the rRNA of the large subunit rather than by ribosomal proteins, establishing the ribosome as a ribozyme. [1, 26, 77, 81, 86] Conserved rRNA sequence has been shown to participate directly in tRNA selection, tRNA and mRNA binding, translocation, and peptidyl transfer, further highlighting the centrality of rRNA to ribosomal function. [77, 81, 86]

Functionally, ribosomes provide defined binding sites for mRNA and tRNAs, translocate stepwise along the mRNA, and decode one codon at a time, assembling the corresponding amino acids into a growing polypeptide chain. [26, 77] The small subunit is primarily responsible for decoding, while the large subunit catalyses peptide bond formation at the peptidyl transferase centre. [77, 81] In this context, ribosomal proteins play crucial but largely supportive roles, stabilising rRNA structure, modulating flexibility, and participating in regulatory interactions rather than acting as primary catalysts. [79, 81, 86]

Extensive sequencing of rRNAs across phylogenetically diverse organisms has produced large comparative databases of rRNA sequences and structural models. [26, 77] These resources have established rRNA as a cornerstone for evolutionary and phylogenetic analyses and as a model system for dissecting structure–function relationships in large RNA–protein complexes. [26, 77]

Chemical modifications of rRNA and functional fine-tuning

Landscape and essential roles of rRNA modifications

rRNA represents one of the most heavily modified classes of cellular RNA. In humans, rRNAs contain more than 210 internal modification sites, including 2'-O-methylations, pseudouridines, and multiple types of base methylations. [32, 83] These chemical modifications are non-randomly distributed and frequently cluster in functionally important regions of the ribosome, such as the peptidyl transferase centre and the decoding site. [83] Ribosomal RNAs thus provide a particularly striking example of how chemical modifications support RNA function: the absence of internal pseudouridines and 2'-O-methylated residues can block rRNA biogenesis and prevent the assembly of functional ribosomes. [32, 79]

Beyond their architectural contributions, rRNA modifications constitute a key layer of post-transcriptional regulation that helps preserve rRNA conformation, stabilise specific base-pairing and tertiary contacts, and optimise ribosomal performance. [32, 83] By fine-tuning the translational apparatus, these modifications safeguard the accuracy and efficiency of protein synthesis and thereby support critical cellular processes, including stress responses, cell differentiation and genomic stability. [83]

rRNA epitranscriptomics in disease and cancer

Dysregulation of rRNA modifications has emerged as an important factor in human disease, particularly cancer. [79, 83, 87] Abnormal patterns or levels of specific modifications can perturb ribosome structure and function, reprogramming translation to favour the synthesis of proteins that promote malignant phenotypes. Distinct modification signatures in cancer cells have been associated with

enhanced production of proteins that drive proliferation, invasion and metastasis, as well as with the emergence of chemoresistance through preferential translation of resistance-related factors. [83]

Organisation of rRNA genes and nucleolar architecture

rDNA repeats and transcription of pre-rRNAs

Ribosome biogenesis is a major cellular enterprise that is tightly coordinated in space and time. [80, 84, 87, 88, 89] In eukaryotes, nucleoli form around nucleolar organising regions (NORs), which contain hundreds of tandem repeats of ribosomal DNA (rDNA) encoding pre-rRNAs. [80, 88] In humans, these rDNA genes are located on the short arms of chromosomes 13, 14, 15, 21 and 22, arranged in head-to-tail palindromic repeats. [80, 84] They encode a large polycistronic precursor—typically denoted 45S or 47S pre-rRNA—that contains the sequences for 18S, 5.8S and 28S rRNAs, interspersed with external and internal transcribed spacers (ETS and ITS). [79, 80, 84]

The transcription of this precursor is carried out by RNA polymerase I (Pol I) within the nucleolus and accounts for the majority of cellular RNA synthesis. [79, 88] Several hundred copies of the 5S rDNA gene, located on chromosome 1 within the nucleoplasm, provide the independently transcribed 5S rRNA that will ultimately join the LSU. [84] The very high rDNA copy number and robust Pol I transcription are necessary to sustain production of up to 10^7 ribosomes per cell, ensuring a sufficiently large ribosome pool for rapidly proliferating cells. [78, 79, 80]

Processing, modification and assembly of ribosomal subunits

Ribosome biogenesis proceeds through a series of tightly regulated events, spanning rDNA transcription, pre-rRNA processing, rRNA modification, assembly with ribosomal proteins, and nucleo-cytoplasmic export of maturing subunits. [79, 82, 84, 85, 87, 89]

This maturation is supported by a large ensemble of auxiliary factors. More than 200 non-ribosomal proteins and around 75 small nucleolar RNAs (snRNAs) are required for proper processing, folding, modification and quality control of pre-rRNAs. [84, 87, 89]

After nuclear assembly and checkpoint control, pre-40S and pre-60S subunits are exported to the cytoplasm, where final maturation events occur. [84, 85] The fully competent 40S and 60S subunits then join, together with mRNA and initiator tRNA, to form the 80S pre-initiation complex, poised to initiate translation. [77, 84]

rRNA in cellular homeostasis and pathology

As the scaffold and catalytic heart of the ribosome, rRNA is indispensable for ribosome synthesis, assembly and function. [77, 79, 83, 87] Because protein synthesis lies at the centre of cell growth, division and differentiation, perturbations in rRNA transcription, processing, modification or ribosomal assembly are tightly linked to human disease. [79, 83, 87] Defects in these processes manifest as congenital ribosomopathies, while adaptive or pathological rewiring of rRNA transcription and modification contributes to oncogenesis and tumour progression. [79, 83]

Deciphering the molecular mechanisms that govern rRNA biogenesis and epitranscriptomic regulation, and understanding how their dysregulation alters translational output, are therefore central to elucidating the role of ribosomes in normal

physiology and disease. [77, 79, 83, 87] In this context, rRNA and its associated processing and modifying machineries are emerging as critical regulatory hubs and promising targets for novel therapeutic strategies. [79, 83, 87]

1.1.6 Influence of intercalation on DNA and RNA

The effect of intercalation on DNA transcription

DNA intercalation is a mode of ligand–DNA interaction in which planar aromatic molecules insert between adjacent base pairs of the DNA double helix. This process produces substantial structural and topological changes in DNA and, consequently, can strongly affect transcription. Since transcription requires controlled DNA accessibility, correct chromatin organisation, recruitment of transcription factors, and the coordinated activity of RNA polymerases, even local distortions caused by intercalating compounds may have broad consequences for gene expression.

At the structural level, intercalation increases the distance between neighbouring base pairs, unwinds the DNA helix, stiffens the duplex, and may alter DNA melting behaviour. These changes can affect the ability of transcription-related enzymes and regulatory proteins to recognise, bind to, and process DNA. By altering the geometry and flexibility of the double helix, intercalators may interfere with transcription complex formation, reduce promoter accessibility, or impede RNA polymerase progression along the template. [90, 91]

One of the major consequences of DNA intercalation is inhibition of RNA polymerase activity. Several intercalating agents have been shown to cause eviction or degradation of RNA polymerases I and II from chromatin. Importantly, this effect does not always require direct DNA damage, indicating that distortion of DNA structure and chromatin organisation alone may be sufficient to impair transcription. RNA polymerase I appears particularly sensitive to intercalators, and ribosomal RNA synthesis is often inhibited at lower drug concentrations than those required to affect RNA polymerase II- or RNA polymerase III-dependent transcription. This sensitivity is especially relevant because rRNA synthesis is essential for ribosome biogenesis and cellular growth. [92, 93, 94, 95]

Intercalators may also affect transcription indirectly through chromatin remodelling. Their binding to DNA can destabilise nucleosomes, evict histones, trap topoisomerases, and promote the formation of non-canonical DNA structures such as Z-DNA. These effects can lead to extensive changes in chromatin accessibility and topology. In actively transcribed regions, the accumulation of topological stress, including altered supercoiling, may facilitate further intercalator binding and contribute to the collapse of transcriptional machinery. Thus, intercalation not only disturbs the DNA helix locally but may also propagate larger-scale changes in chromatin structure. [92, 93, 96]

Another important mechanism involves interference with transcription factor binding. Some intercalators prevent sequence-specific transcription factors, such as Sp1, from binding their recognition sites. Others can displace transcription factors from pre-existing DNA–protein complexes. Because transcription factors are required for promoter recognition, polymerase recruitment, and regulation of transcriptional output, their displacement can result in promoter-specific inhibition of gene expression. [97, 98, 99]

The effect of intercalation may differ depending on the stage of transcription. Some intercalators primarily inhibit transcription initiation by destabilising pre-initiation complexes or preventing the recruitment of essential regulatory proteins. Others affect transcription elongation by arresting elongating RNA polymerases

or inducing premature termination. Therefore, intercalation can interfere with transcription at multiple mechanistic levels, from promoter activation to productive RNA synthesis. [93, 95, 100]

Although the dominant effect of DNA intercalation is transcriptional repression, the outcome is not always uniform. The response depends strongly on promoter architecture, local DNA sequence, chromatin context, supercoiling state, and the chemical structure of the intercalating compound. Some promoters are highly sensitive to intercalators, whereas others remain relatively resistant. In certain contexts, low concentrations of intercalating agents may even stimulate transcription, as observed for some viral genes, possibly by modulating host RNA polymerase II activity. [101, 102]

In addition to general transcription inhibition, some intercalators can produce selective or regulatory effects. Certain compounds may rapidly reactivate silenced genes by influencing DNA methyltransferases or modifying chromatin marks. Furthermore, designed bis-intercalators and polyamide–intercalator conjugates can be engineered to target specific DNA sequences or genomic regions, allowing more selective modulation of transcription. This sequence-directed approach highlights the potential of intercalators not only as broadly cytotoxic agents but also as tools for targeted control of gene expression. [103, 104]

Overall, DNA intercalation has profound consequences for transcription. By distorting the DNA helix, altering chromatin architecture, changing DNA topology, displacing transcription factors, and disrupting RNA polymerase function, intercalating compounds can strongly suppress gene expression. The most pronounced inhibitory effects are often observed for RNA polymerase I-dependent ribosomal RNA synthesis, although RNA polymerase II- and III-mediated transcription can also be affected. At the same time, the transcriptional response to intercalation is context-dependent and may include promoter-specific inhibition, selective gene regulation, or, in some cases, transcriptional activation. These observations demonstrate that intercalation is not simply a passive DNA-binding event, but a powerful mechanism capable of reshaping transcriptional regulation. [90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 105, 106]

The effect of intercalation on translation from RNA

Intercalation, defined as the insertion of planar molecules between stacked nucleic acid bases, can significantly influence RNA structure and function. Although intercalation is most commonly discussed in relation to DNA, similar interactions may also occur with RNA or RNA-containing complexes, thereby affecting translation. Since translation depends on mRNA structural accessibility, ribosome recruitment, codon reading, translocation, and ribosome biogenesis, intercalating compounds may interfere with protein synthesis at multiple levels. [95, 107, 108, 109, 110, 111]

One of the best-characterised mechanisms by which intercalators inhibit translation involves antisense oligonucleotides. Several studies have shown that covalent attachment of intercalating agents, such as acridine derivatives, to oligonucleotides increases their affinity for complementary mRNA sequences. This enhanced binding stabilises mRNA–oligonucleotide hybrids and improves the ability of antisense molecules to block translation. As a result, intercalator-conjugated oligonucleotides can inhibit protein synthesis more efficiently than non-intercalating

analogues. [107, 108, 112]

Intercalating agents may also suppress translation by targeting structured RNA elements required for ribosome recruitment. This mechanism is particularly relevant for viral RNAs that initiate translation via internal ribosome entry sites (IRES elements). Compounds such as quinacrine have been shown to inhibit IRES-mediated translation in viruses, including hepatitis C virus, encephalomyocarditis virus, and poliovirus. In these systems, the degree of translation inhibition depends on the intercalator’s affinity for specific RNA structures within the IRES region. By binding to these structured elements, intercalators may disturb RNA conformation and prevent efficient ribosome recruitment or initiation complex formation. [109, 110, 113, 114, 115]

The effect of intercalation on translation is not limited to artificial or drug-induced interactions. Structural studies indicate that natural base intercalation events occur within the ribosome itself. For example, specific ribosomal RNA bases, such as A1503 in 16S rRNA, can intercalate between mRNA bases during translocation. This type of interaction maintains the translational reading frame and helps prevent frameshift errors. Therefore, intercalation can have two opposite functional meanings in translation: artificial intercalation by small molecules may inhibit protein synthesis, whereas controlled intercalation within the ribosome can support translational fidelity. [109]

Intercalators may also indirectly affect translation by interfering with processes required for ribosome production. Some DNA/RNA intercalating compounds inhibit transcription initiation, particularly RNA polymerase I-dependent transcription, which is responsible for rRNA synthesis. They may also disturb rRNA processing, alter chromatin organisation, or affect the expression of genes involved in ribosome biogenesis. Since ribosome production is essential for cellular translation capacity, such effects may reduce protein synthesis even in the absence of direct binding to mature mRNA. [95, 101, 116, 117]

The consequences of intercalation are therefore highly context-dependent. In synthetic antisense systems, intercalators can be deliberately used to strengthen hybrid formation and enhance translation blockage. In viral models, they may inhibit translation by disturbing structured RNA elements required for IRES-dependent initiation. In ribosomes, natural intercalation events may be necessary for accurate decoding and maintenance of the reading frame. Moreover, the sensitivity of a given transcript or translation system may depend on RNA sequence, secondary structure, accessibility, and the chemical properties of the intercalating compound. [95, 101, 109, 110, 111]

These mechanisms make intercalators attractive tools for gene regulation and antiviral therapy. Intercalator-conjugated oligonucleotides may improve antisense-based inhibition of selected mRNAs, while small molecules that bind viral IRES structures may suppress viral protein synthesis. However, their therapeutic development requires careful evaluation of specificity. Because many intercalators can also affect DNA structure, transcription, rRNA synthesis, and chromatin organisation, off-target effects may contribute to toxicity or unintended changes in gene expression. [92, 112, 114]

Overall, intercalation can modulate translation from RNA through both direct and indirect mechanisms. Direct effects include stabilisation of antisense oligonuc-

leotide-mRNA hybrids and disruption of RNA structures required for ribosome recruitment or translation initiation. Indirect effects include inhibition of transcription, rRNA processing, and ribosome biogenesis. Although most experimentally observed effects of artificial intercalators are inhibitory, natural intercalation within the ribosome can support accurate translation. Thus, intercalation represents a versatile mechanism capable of suppressing, regulating, or stabilising different stages of RNA-dependent protein synthesis. [92, 95, 101, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117]

Impact of translation/transcription kinetics disrupted by intercalators in targeted diseases or therapies

The disruption of transcription and translation kinetics by intercalating compounds has major implications for disease treatment, particularly in oncology and antiviral therapy. DNA intercalators, including doxorubicin, actinomycin D, aclarubicin, BMH-21, and various synthetic or natural molecules, insert between stacked base pairs and modify the physical properties of nucleic acids. As a result, they can interfere with essential biological processes, including DNA replication, transcription, RNA processing, ribosome biogenesis, and translation. Because rapidly dividing cells and viruses rely heavily on efficient nucleic acid metabolism and protein synthesis, these processes represent attractive therapeutic targets. However, the same mechanisms that make intercalators effective also pose significant challenges, including toxicity, resistance, and limited selectivity. [92, 94, 95, 105]

In cancer therapy, one of the most important consequences of intercalation is the inhibition of transcription, particularly of ribosomal RNA. Many tumour cells display increased ribosome biogenesis to support rapid growth and proliferation. Therefore, disruption of RNA polymerase I activity and rRNA production can trigger nucleolar stress, impair ribosome assembly, and activate apoptotic pathways. This mechanism is especially relevant for intercalators that preferentially affect RNA polymerase I-dependent transcription. For example, BMH-21 inhibits RNA polymerase I and induces degradation of the polymerase complex without necessarily activating a classical DNA damage response, thereby potentially reducing some genotoxic side effects. [92, 94, 95, 105]

The therapeutic value of transcriptional disruption is closely linked to cancer cells' dependence on highly active gene-expression programs. Tumours driven by short-lived oncogenic proteins, such as MYC, may be particularly vulnerable to interference with transcription or translation kinetics. Since proteins with rapid turnover require continuous synthesis to maintain oncogenic signalling, inhibition of their production can produce strong anti-tumour effects. Targeting translation or ribosome function in combination with DNA intercalation is therefore being explored as a strategy for difficult-to-treat cancers and therapy-resistant tumours. [118]

Intercalators may also influence translation more directly or indirectly. Direct effects can involve binding to structured RNA regions, stabilisation of nucleic acid hybrids, or interference with ribosome recruitment. Indirect effects result from impaired rRNA synthesis, defective ribosome biogenesis, and altered expression of translation-related genes. In this way, intercalators can reduce the cell's overall translational capacity. Since malignant cells often require increased protein synthesis to sustain proliferation, metabolic adaptation, and stress responses, disruption

of translation kinetics may contribute substantially to the cytotoxic activity of intercalating agents. [95, 105, 118]

Beyond cancer, intercalators are being investigated for antiviral applications. Some plant-derived and synthetic intercalating compounds can inhibit viral replication by stabilising viral nucleic acid structures, interfering with genome expression, or blocking structured RNA elements required for translation initiation. This is particularly relevant for viruses that use internal ribosome entry sites, such as the hepatitis C virus, where intercalator binding to IRES structures can suppress viral protein synthesis. Similar principles have been discussed for other viral infections, including SARS-CoV-2, although antiviral applications remain less established than anti-cancer uses and require further validation. [114, 119]

A major direction in the development of intercalator-based therapies is improved specificity. Classical intercalators often act broadly because DNA and RNA are present in all cells and because transcription and translation are essential universal processes. To overcome this limitation, newer strategies focus on molecules that selectively recognise disease-relevant nucleic acid sequences, secondary structures, or chromatin environments. Engineered intercalators, sequence-selective conjugates, and structure-targeted compounds may enable more precise inhibition of oncogenic or viral gene expression programs while sparing normal cells. [114]

Drug delivery systems represent another important strategy for increasing therapeutic selectivity. Nanoparticle-mediated delivery can enhance the accumulation of intercalating agents in tumour tissue, improve intracellular delivery, and reduce systemic exposure. Such platforms may decrease adverse effects while maintaining or increasing anti-tumour efficacy. In addition, combination therapies pairing intercalators with agents that affect ribosome function, translation control, DNA repair, or cell-survival pathways may produce synergistic effects, particularly in resistant cancers. [118, 120, 121]

Despite their therapeutic potential, intercalators remain a double-edged sword. Since transcription and translation are essential in both diseased and healthy cells, broad disruption of these processes can cause serious off-target toxicity. This is especially relevant for highly proliferative normal tissues, such as bone marrow and gastrointestinal epithelium, as well as for organs sensitive to specific drugs, such as the heart with anthracyclines. Toxicity may result not only from direct DNA damage but also from chromatin disruption, impaired RNA polymerase function, altered rRNA synthesis, and global changes in gene expression. [94, 122]

Resistance is another major limitation. Cancer cells may reduce intercalator accumulation through altered uptake or increased efflux, activate compensatory survival pathways, change DNA repair capacity, or remodel transcriptional and translational programs. Endogenous molecules may also influence therapeutic response. For example, polyamines such as spermine can modulate drug-DNA interactions and thereby affect sensitivity to intercalator-induced transcriptional inhibition. These factors complicate treatment outcomes and highlight the need to understand not only drug binding but also cellular context. [122]

Overall, disruption of transcription and translation kinetics by intercalators is central to the therapeutic activity of many anti-cancer agents and represents a promising strategy for antiviral and gene-regulatory applications. By inhibiting RNA polymerases, impairing rRNA synthesis, disturbing chromatin archi-

ture, blocking structured RNA elements, and reducing ribosome biogenesis or translational output, intercalators can strongly suppress disease-associated gene expression. However, their clinical usefulness depends on balancing potency with selectivity. Current research is therefore moving toward rationally designed intercalators, sequence- or structure-selective compounds, nanoparticle-based delivery systems, and combination therapies that maximise therapeutic efficacy while reducing toxicity and overcoming resistance.[92, 94, 95, 105, 114, 118, 119, 120, 122]

The key to understanding all these processes

The biological consequences of intercalation cannot be fully understood by considering intercalators as static molecules bound to nucleic acids. Instead, their effects on transcription, translation, chromatin organisation, ribosome biogenesis, and therapeutic activity arise from continuous, dynamic interactions with DNA, RNA, and nucleoprotein complexes. Intercalation is therefore not a single binding event, but a process in which association, dissociation, redistribution, and competition with cellular proteins collectively determine the final biological outcome.

The relationship between intercalators and nucleic acids is also concentration- and time-dependent. Low levels of binding may produce subtle changes in nucleic acid structure, sometimes leading to selective or context-dependent effects, including altered promoter activity or stimulation of specific viral transcriptional programs. Higher levels of binding can cause broader disruption of DNA topology, chromatin organisation, polymerase progression, and RNA processing. This explains why intercalators may act selectively under some conditions but produce global cytotoxicity under others. Their biological activity depends not only on whether they bind DNA or RNA, but also on how long they remain bound, how frequently they dissociate, which structures they prefer, and whether cellular machinery can repair or compensate for the disturbance. [92, 95, 101, 102]

This dynamic view is particularly important in therapeutic contexts. In cancer, intercalators exploit the dependence of tumour cells on intense transcription, rapid ribosome biogenesis, and sustained protein synthesis. By disrupting RNA polymerase activity, rRNA synthesis, chromatin structure, or translation-related pathways, they can induce nucleolar stress, suppress oncogenic gene-expression programs, and trigger apoptosis. However, because transcription and translation are essential in normal cells as well, therapeutic success depends on achieving a favourable balance between potency and selectivity. This balance is determined by the kinetics of intercalator–nucleic acid interactions: binding affinity, residence time, sequence or structure preference, intracellular concentration, and accessibility of target regions. [92, 94, 105, 118, 122]

The same concept applies to antiviral strategies. Viral genomes and transcripts often contain structured RNA or DNA regions that are essential for replication, transcription, or translation. Intercalators that preferentially bind these regions may inhibit viral gene expression by blocking nucleic acid processing or ribosome recruitment. However, effective antiviral application requires discrimination between viral and host nucleic acids. This again highlights the importance of understanding the detailed interaction kinetics between intercalators and specific DNA or RNA structures. [110, 114, 119]

Resistance and toxicity also arise from the dynamic nature of these interactions. Cells may reduce drug accumulation, increase efflux, activate compensatory

transcriptional or translational pathways, alter chromatin organisation, or modulate nucleic acid-binding environments through endogenous molecules such as polyamines. These factors change the effective interaction between intercalators and their nucleic acid targets. Therefore, resistance is not only a property of the drug or the cell alone, but of the changing relationship between the intercalator, DNA/RNA structure, chromatin context, and cellular response mechanisms. [122]

For this reason, the future development of intercalator-based therapies depends on moving beyond general descriptions such as “DNA-binding” or “RNA-binding.” A more precise understanding requires analysis of binding constants, association and dissociation rates, residence times, sequence preference, structural selectivity, and effects on nucleic acid mechanics. Single-molecule approaches, fluorescence-based methods, structural studies, and kinetic analyses are particularly valuable because they can reveal how individual intercalator molecules interact with DNA or RNA over time. Such approaches may help distinguish compounds that cause broad nucleic acid disruption from those that selectively modulate disease-relevant structures. [104, 120, 121]

Overall, the key to understanding the effects of intercalators on transcription, translation, and therapy lies in analysing their constant interactions with DNA and RNA. Intercalators influence biological systems not simply because they bind nucleic acids, but because their binding continuously reshapes nucleic acid structure, accessibility, topology, and protein interactions. These molecular events determine whether the outcome is transcriptional repression, translation inhibition, ribosome biogenesis failure, antiviral activity, cytotoxicity, or therapeutic benefit. Understanding the kinetics and specificity of intercalator–DNA/RNA interactions is therefore essential for explaining their biological effects and for designing safer, more selective therapeutic agents.

1.2 Cyanine dyes YO-PRO-1 and YOYO-1

Fluorescence probes in contemporary nucleic-acid research

Fluorescence techniques have been extensively investigated and widely applied across basic biological, biomedical, and clinical research. The field continues to advance rapidly, focusing on the development of new fluorophores with improved photophysical properties and enhanced biocompatibility, as well as the design of novel probes with greater specificity and higher signal-to-noise ratios. Additionally, ongoing efforts aim to create more efficient and precise analytical methods. As a result, expectations and demands for fluorescence-based technologies continue to grow within the scientific community.

A fluorescent probe is a fluorophore engineered to accumulate in a specific region of a biological specimen or to respond to a defined stimulus. These probes have been widely used for almost a century to investigate cellular processes because of their remarkable sensitivity and selectivity. Their use has become even more prominent as concerns about the safety and environmental risks of radioactive probes have increased. In parallel, the growing reliance on cellular assays has further driven the popularity and application of fluorescent probes in biological research. [123] Fluorescent probes can take different forms, including genetically encoded probes such as fluorescent proteins, fluorescently labelled proteins or peptides, or small molecule probes. Moreover, these probes can be engineered for use in a wide variety of assay modalities, allowing flexibility in their application for studying biological systems. [123]

Cyanine dyes: structural definition and tunable photophysics

Cyanine dyes, first described over 150 years ago, are characterised by a molecular structure consisting of two heterocyclic units linked by a polymethine bridge containing an odd number of carbon atoms. [124] By modifying the nature of the heterocycles and the length of the bridge, their absorption and emission properties can be tuned to cover a broad range of the visible and near-infrared spectrum. [124]

Cyanine dyes are notable for their high molar extinction coefficients (typically exceeding $10^4 \text{ M}^{-1}\text{cm}^{-1}$) and moderate fluorescence quantum yields, which together have led to their widespread use in diverse applications such as photosensitizers, biological stains, fluorescent labels, and molecular probes. [124]

Cyanine dyes, long known for their role in the photographic industry, have more recently gained importance in biological research, particularly for staining duplex (double-stranded) DNA. [125] Cationic cyanine dyes display dramatic fluorescence enhancement when bound to nucleic acids. Moreover, covalently linking two cyanine dyes to create a bichromophore significantly increases their binding affinity for nucleic acids by nearly two orders of magnitude, similar to what has been observed for phenanthridium dyes such as ethidium bromide. [125]

Monomeric and dimeric cyanine dyes differ in their binding modes, photophysical responses, and selectivity for nucleic acid structures. Dimeric dyes often provide higher affinity and unique selectivity, but their aggregation can quench fluorescence unless disrupted by specific binding events. These differences are crucial for applications in bioimaging, sensing, and nucleic acid detection. [125, 126, 127, 128, 129, 130, 131]

Oxazole yellow cyanines as DNA intercalators: YO-PRO-1 and YOYO-1

YO-PRO-1 (Fig. 7) is one of many DNA staining dyes available on the market. It has a flat aromatic structure consisting of benzoxazole and quinoline rings connected with the cyanine-based linker. It is known as a double-strand DNA fluorescent intercalator. The dye is non-fluorescent in the free state and emits green light spectra after complex formation (Fig. 8).

YOYO-1 (Fig. 9) is a homodimer of YO-PRO-1 dye. It has two aromatic moieties, resulting in two different modes of reaction (Fig. 10), mono-intercalation and bis-intercalation. The dye is non-fluorescent in the free state and emits a green spectrum upon complex formation.

The synthesis of oxazole yellow (YO-PRO-1) cyanine dyes and their homodimeric forms (YOYO-1) marked a significant breakthrough in molecular biology, enabling fluorescence detection of DNA with sensitivity comparable to that of radioactive probes. [132] Due to their strong photophysical properties and high DNA-binding affinity, cyanine fluorescent dyes have proven especially valuable for DNA detection across a wide range of techniques, including polymerase chain reaction, restriction fragment sizing, and flow cytometry. They have also enabled extremely high sensitivity, allowing for size determination and imaging of individual DNA molecules labelled with these dyes. [133] YOYO-1 is among the most widely used fluorophores for DNA staining and, owing to its minimal background fluorescence, has become a powerful probe for DNA quantification, visualisation of DNA during electrophoresis, characterisation of DNA–ligand interactions, and single-molecule imaging. [134]

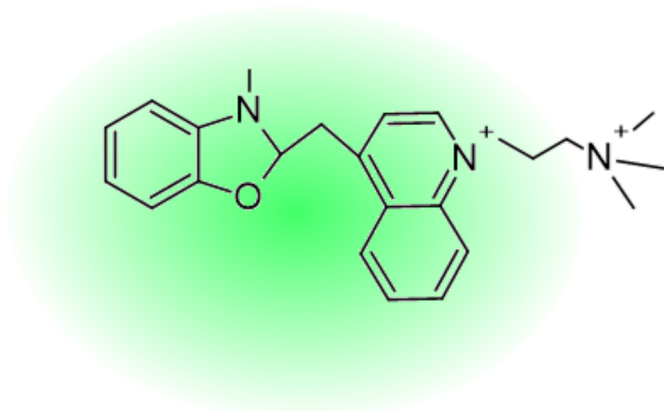


Fig. 7: 2D structure of YO-PRO-1 dye. The dye consists of benzoxazole and quinoline rings connected with a cyanine-based linker. The green area marks the aromatic structure responsible for fluorescence. The structure was created in ChemSketch.



Fig. 8: Scheme of YO-PRO-1-DNA complex formation. YO-PRO-1 intercalates between base pairs in the DNA helix. The binding size is 5 bp. After complex formation, the non-fluorescent dye (in a free state) emits green fluorescence ($\lambda = 509$ nm).

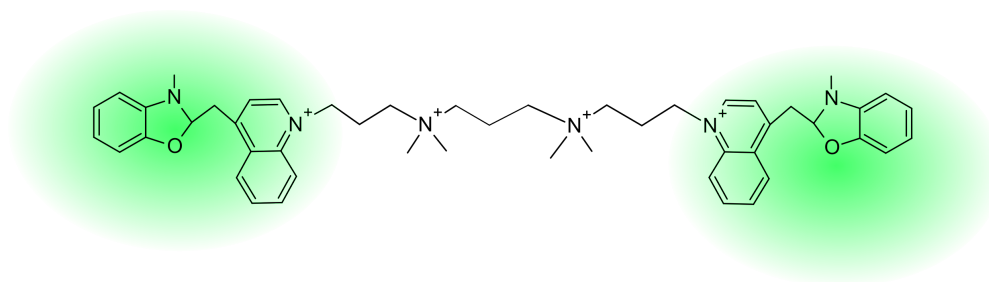


Fig. 9: 2D structure of YOYO-1 dye. The dye consists of benzoxazole and quinoline rings connected with a cyanine-based linker. The dye has two aromatic moieties. The green area marks the aromatic structure responsible for fluorescence. The structure was created in ChemSketch.

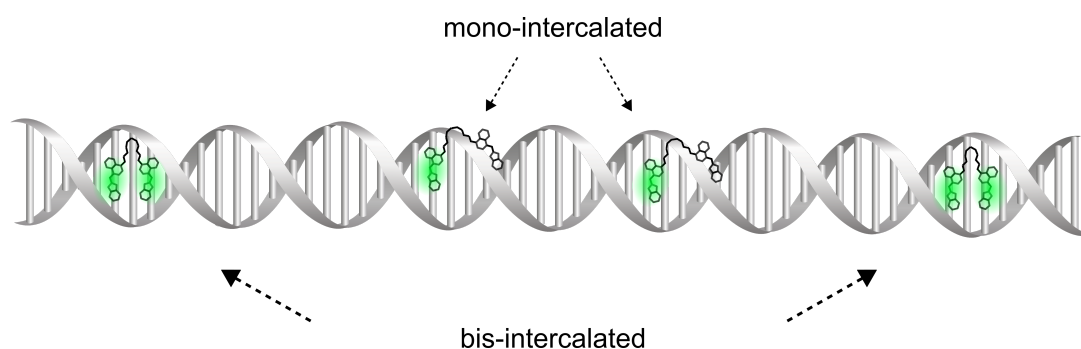


Fig. 10: Scheme of YOYO-1-DNA complex formation. YOYO-1 intercalates into the DNA helix in between base pairs. The binding size is 3.5 bp. After complex formation, the non-fluorescent dye (in a free state) emits green fluorescence ($\lambda = 509$ nm).

Binding modes of cyanine dyes to DNA

Intercalation as a general binding mechanism

An important class of DNA-binding ligands are intercalators, which interact with DNA by inserting aromatic moieties between adjacent base pairs. This mode of binding disrupts the regular stacking of the DNA helix, causing local unwinding and lengthening of the duplex. Understanding both the dynamic behaviour and the equilibrium properties of DNA-intercalator complex formation is essential for optimising their binding characteristics for specific biological or therapeutic applications, such as anticancer drug design, molecular probing, and gene regulation. [90]

Intercalation stabilises the double-stranded DNA (dsDNA) helix by enhancing base stacking interactions and reducing helical flexibility, often resulting in local unwinding and lengthening of the DNA. [90] A single DNA intercalation event can induce significant structural alterations in the DNA helix, including helix unwinding that may exceed 50% of its native twist and substantial elongation, with near doubling of the base-pair spacing at the intercalation site. [90] Although DNA helix unwinding accompanies intercalation — compensating for helix extension and enhancing base-intercalator stacking interactions — the DNA-ligand complex preserves key structural features of native DNA; notably, the distance between adjacent phosphate groups remains nearly invariant, maintaining the continuity of the backbone. [90]

Intercalation involves noncovalent π - π stacking interactions between an intercalating molecule and the π -orbitals of adjacent DNA base pairs, often accompanied by hydrogen bonding. This mode of binding extends the DNA helix and often

partially unwinds the double helix. [135] Such structural alterations can influence higher-order chromatin organisation, particularly by modifying nucleosome structure and DNA accessibility in transcription, replication, and repair. [135, 136]

YO-PRO-1 and YOYO-1: monointercalation, bisintercalation and groove binding

YOYO-1 and YO-PRO-1 are oxazole yellow dyes that bind to DNA via intercalation and are widely employed in single-molecule fluorescence imaging of DNA. In free solution, these dyes are almost nonfluorescent, but upon binding to DNA, their fluorescence quantum yield increases dramatically, by several orders of magnitude. YO-PRO-1 is a mono-intercalator containing a single aromatic moiety, while YOYO is a bis-intercalator composed of two aromatic moieties linked together, with each moiety intercalating between base pairs and leaving one base pair unoccupied between the two sites. [137]

YOYO-1 intercalates both of its chromophores between base pairs, typically spanning two sites on the DNA (bis-intercalation). [137, 138] This process can occur in two steps: initial mono-intercalation followed by full bis-intercalation, with each step having distinct equilibrium constants. [138] Therefore, one equilibrium constant is provided in the literature (Table 5). YO-PRO-1 intercalates a single chromophore between adjacent base pairs (monointercalation) [137, 138, 139], with the equilibrium constant reported to be $10^5 - 10^6$ (Table 6).

Consistent with these mechanisms, YOYO-1 bis-intercalation causes pronounced elongation of the DNA helix (commonly reported as up to $\sim 30\text{-}35\%$ increase in contour length at saturation) and induces DNA unwinding or underwinding. [137, 140, 141, 142, 143] In contrast, monointercalation by YO-PRO-1 produces smaller structural changes (e.g. $\sim 6\%$ contour-length increase) and less pronounced unwinding. [137, 140] These mechanistic differences underlie distinct kinetics: YOYO-1 binds and dissociates substantially more slowly than YO-PRO-1. [137, 138]

Both dyes primarily interact with DNA via intercalation, but at high concentrations or under certain conditions, non-specific electrostatic or groove binding may occur, and both can exhibit minor groove binding depending on the base-pair-to-dye ratio. [138, 144, 145] At high base pair-to-dye ratios (low dye relative to DNA), intercalation dominates, whereas at low base pair-to-dye ratios (high dye loading), minor groove binding can become favoured. [144, 146] These alternative binding modes restrict dye motion less effectively than intercalation and can therefore yield lower fluorescence enhancement. [144, 147]

Table 5: Reported in literature equilibrium constant values of the reaction between YOYO-1 dye and DNA.

K [M ⁻¹]	Method	Reference
$3.875 \pm 0.128 \times 10^6$	Optical tweezers	[137]
1.4×10^8	Magnetic tweezers	[140]
$8 \pm 4 \times 10^8$	AFM	[142]
$3.58 \pm 0.103 \times 10^6$	Magnetic tweezers	[143]
$3 \pm 2 \times 10^7$	Fluorescence titration	[146]
$2.8 \pm 1.4 \times 10^4$	Optical tweezers	[148]
6.0×10^8	Absorption spectroscopy	[149]
4.9×10^6	Fluorescence titration	[150]

Table 6: Reported in literature equilibrium constant values of the reaction between YO-PRO-1 dye and DNA.

K [M ⁻¹]	Method	Reference
$7.9 \pm 0.2 \times 10^5$	Absorption spectroscopy	[133]
$5.78 \pm 0.80 \times 10^5$	Optical tweezers	[137]
$2.8 \pm 0.4 \times 10^5$	Fluorescence titration	[146]
$2.9 \pm 0.9 \times 10^5$	Optical tweezers	[148]

Stoichiometry, non-cooperativity and ionic strength dependence

YOYO-1 and YO-PRO-1 differ in their DNA binding stoichiometry: YOYO-1 binds at approximately 1 dye per 3.5-4 base pairs (Table 7), whereas YO-PRO-1 binds at approximately 1 dye per 3-5 base pairs (Table 8). [137, 138] Both dyes bind DNA in a non-cooperative manner: YOYO-1 binding is stepwise but independent, while YO-PRO-1 is well described by a simple, non-cooperative model. [137, 138, 151]

Cyanine dyes bind to DNA less strongly at elevated ionic strength. [140] For YOYO-1 and YO-PRO-1, ionic strength strongly influences equilibrium constants and complex stability, and at high ionic strength, both dyes can be rapidly removed from DNA. [151] The ionic sensitivity is more pronounced for YOYO-1 and has been attributed to its higher positive charge (+4) compared with YO-PRO-1 (+2), which enhances electrostatic interactions with the negatively charged DNA backbone while simultaneously increasing susceptibility to charge screening. [151]

Table 7: Reported in literature size of the binding site of YOYO-1 dye.

n	Method	Reference
3.2 ± 0.6	Magnetic tweezers	[140]
3.1 ± 0.1	AFM	[142]
3.22 ± 0.30	Magnetic tweezers	[143]
4.7 ± 0.7	Optical tweezers	[148]

Table 8: Reported in literature size of the binding site of YO-PRO-1 dye.

n	Method	Reference
3 ± 1	Circular dichroism	[133]
3.8 ± 1.0	Optical tweezers	[148]

Mechanisms of fluorescence enhancement upon DNA binding*Restriction of torsional motion and suppression of ultrafast non-radiative decay*

In aqueous solutions, YO-PRO-1 is nearly nonfluorescent; however, its fluorescence quantum yield increases significantly upon binding to DNA, and a comparable enhancement is observed in viscous solvents such as glycerol. [152] This behaviour is attributed to restricted rotational mobility around the polymethine bridge connecting the two aromatic ring systems. When such rotation is hindered by the structured DNA environment or by increased solvent viscosity, non-radiative deactivation pathways are suppressed, leading to enhanced fluorescence emission. [152]

In aqueous solution, free dyes exist in two states: non-aggregated dyes and intramolecular H-type aggregates. [147] For non-aggregated dyes, excited-state dynamics are dominated by rapid nonradiative decay (on the order of ~ 5 ps), linked to large-amplitude motion around the monomethine bridge. [147] When these dyes bind to DNA, strong fluorescence enhancement occurs due to inhibition of this nonradiative decay pathway. [147]

Dimer disruption, excitonic coupling and aggregation effects

For homodimeric cyanines (YOYO-1), fluorescence enhancement additionally involves dissociation of nonfluorescent H-dimers. In aqueous solution, YOYO-1 dyes can form H-dimers via self-aggregation, where excitonic interactions delocalize excitation and split energy levels into a lower-energy dark state and a higher-energy bright state. [132] Upon binding to DNA, these dimers are disrupted, suppressing excitonic interactions and simultaneously restricting large-amplitude motion, thereby allowing fluorescence to become fully active. [132, 144, 147] Loss of exciton coupling upon bis-intercalation is attributed not only to increased chromophore separation but also to their quasi-orthogonal mutual orientation, which prevents self-quenching. [132] Nonetheless, interactions between chromophores can remain strong enough to permit ultrafast excitation energy hopping, as evidenced by rapid fluorescence anisotropy decay. [147]

Photophysical determinants in quantitative fluorescence measurements

The intrinsic photophysical characteristics of YOYO-1 and YO-PRO-1 are crucial in defining their apparent molecular brightness in fluorescence correlation spectroscopy (FCS). [153, 154] In FCS, brightness is primarily determined by a dye’s quantum yield and extinction coefficient; when both are high, each molecule emits more detectable photons. [153] However, transitions into long-lived non-emissive triplet states reduce photon output and lower effective brightness when triplet yields are high. [153] Blinking, arising from stochastic switching between emissive and non-emissive states, similarly reduces average photon output and can distort FCS autocorrelation curves, complicating quantitative interpretation. [154] Variability in photophysical behaviour due to microenvironmental effects or aggregation introduces heterogeneity in brightness distributions that can bias FCS analyses, requiring careful treatment of brightness heterogeneity and dye-specific triplet/blinking susceptibilities for accurate quantification. [153, 154]

More broadly, molecular brightness analysis in systems with multiple brightness states is challenged by overlapping state brightness values, ensemble averaging, shot noise and background, photophysical artefacts (blinking, photobleaching, quenching), and the need for robust calibration and statistically well-posed model selection. [155, 156, 157, 158, 159, 160, 161, 162]

Impact of cyanine intercalation on DNA mechanics and structure

YOYO-1 is widely used to probe the statistical-mechanical properties of DNA, yet its exact impact on DNA rigidity and structure remains uncertain. [140] Single-molecule fluorescence microscopy has enabled detailed studies of DNA transport, stretching under flow or confinement, entropic recoiling, surface diffusion and electrophoretic mobility, frequently using YOYO-1 because of its high association constant and strong fluorescence enhancement. [140] However, intercalating dyes can significantly influence mechanical and structural DNA properties, and systematic data remain limited and often contradictory. [140, 141, 142]

In addition to bending effects, YOYO-1 intercalation causes elongation and untwisting of dsDNA, with contour length increasing linearly with staining ratio up to $\sim 35\%$ relative to native DNA. [140, 148] Despite multiple studies, no consistent consensus has been reached regarding how YOYO-1 alters DNA mechanics, largely due to differences in solution conditions and experimental approaches. [140, 141]

Intercalation can interfere with essential DNA-associated processes, such as transcription, replication, and repair, by altering protein-DNA interactions. [137, 148] In experimental contexts, enzymatic processes can be monitored via displacement of fluorescent intercalators by helicases or dye binding to newly synthesised DNA during polymerase activity, but intercalation-induced structural perturbations can also confound these assays. [148] Accordingly, understanding the mechanisms and kinetics of intercalation is essential for both mitigating unwanted perturbations in biophysical experiments and optimising intercalator-based therapeutics, whose cytotoxicity can derive from precisely such disruptions. [142, 148, 163]

Environmental stability, dissociation and photobleaching

Single-molecule DNA studies frequently require sustained fluorescence signals. YOYO-1 forms a highly fluorescent complex with DNA, but its fluorescence can decrease over time due to photobleaching, with bleaching kinetics depending on excitation power and potentially reflecting different bound states. [164] YO-PRO-1 also shows strong fluorescence when DNA-bound but is generally less resistant to washout, with YOYO-1 being removed from DNA approximately 2-5 times more slowly than YO-PRO-1. [151] Under high ionic strength and buffer flow, dissociation can dominate signal loss for both dyes, with most dye being removed within minutes. [140, 151, 165] Thus, fluorescence decay can be governed primarily by photobleaching in static conditions, whereas dye dissociation dominates under flow or high-salt environments. [151, 164]

A limitation of oxazole yellow dyes is their tendency to induce DNA photocleavage under intense illumination. Both YO-PRO-1 and YOYO-1 can cause single-strand breaks (SSBs), and double-strand breaks (DSBs) detected in microscopy are likely to arise from closely spaced SSBs occurring on opposite strands within ~ 15 bp. [166] This implies that substantial nicking and other damage may precede visible DNA breakage, potentially altering the conformational and dynamic properties of stained DNA prior to overt strand cleavage. [166] Photobleaching mechanisms can differ between free and DNA-bound dyes, and between intercalated and externally bound states, reflecting distinct reactive intermediates and oxygen dependence. [144, 166, 167]

Applications and experimental constraints of cyanine DNA stains

Owing to their negligible background fluorescence, strong fluorescence enhancement upon binding, high binding affinities and large molar absorptivities, oxazole yellow dyes enable pronounced contrast between stained DNA and background, supporting sensitive detection and imaging. [133, 134, 166] YOYO has been widely applied to visualise DNA molecules for measurements of self-diffusion, hydrodynamic stretching, DNA dynamics under confinement and genomic mapping, where its bright signal facilitates contour tracking. [141] Nevertheless, practical limitations remain: for example, although YOYO-1 is efficient and photobleaching-resistant in many contexts, it is impermeable to cell membranes, limiting its ap-

plicability in live- or fixed-cell experiments. [143]

Finally, competitive and contextual effects must be considered in complex biological or experimental environments. Other DNA-binding agents can reduce YOYO-1 and YO-PRO-1 binding via direct competition (e.g. intercalators) or indirect structural modulation (e.g. groove binders altering DNA conformation and mechanics), thereby affecting fluorescence readouts and assay performance. [135, 145, 168] Together, these considerations emphasise that cyanine-dye performance is jointly determined by dye structure (monomer versus dimer), binding-mode distributions (intercalation versus groove binding), environmental parameters (ionic strength, interfaces, flow), and photophysical dynamics (aggregation, triplet formation, blinking and photobleaching). [132, 137, 138, 144, 147, 151, 153, 154]

1.3 Anthracyclines

Chemical and Structural Background

Anthracyclines constitute a major class of antitumor antibiotics and DNA-binding small molecules, ranking among the most potent and extensively studied chemotherapeutic agents used in clinical practice. Originally isolated from mutant strains of the actinobacterium *Streptomyces peucetius*, these compounds have been employed for more than six decades in the treatment of a wide range of malignancies. [169, 170] The earliest members of this class, daunorubicin and doxorubicin, were identified in the 1960s and rapidly demonstrated strong anticancer activity, leading to their widespread clinical adoption. [171] Today, anthracyclines remain a cornerstone of modern chemotherapy and are included in multiple first-line treatment regimens for lymphomas, breast cancer, sarcomas, and leukemias. [172] Owing to their strong DNA intercalation properties, they are regarded as some of the most thoroughly characterised small-molecule intercalators. [173] Despite their long-standing clinical success, their utility is significantly constrained by the development of drug resistance and severe dose-limiting cardiotoxicity. [173]

Among clinically validated anthracyclines, the most widely used compounds are daunorubicin (DAU) and its semisynthetic derivatives doxorubicin (DOX), epirubicin (EPI), and idarubicin (IDA) (Fig. 11-14). [174, 175] Doxorubicin is a prominent member of this family and remains one of the most effective anticancer agents in clinical use. Since its discovery, it has attracted sustained research interest; however, despite extensive investigation, its precise molecular mechanism of action has not yet been fully resolved. The prevailing view is that doxorubicin primarily exerts its anticancer effects through inhibition of human type IIA topoisomerase, acting both as a topoisomerase poison by stabilising the cleavage complex and as a catalytic inhibitor. Increasing evidence indicates that its characteristic modes of DNA binding are central to its biological activity. [176]

Anthracyclines are widely used as broad-spectrum chemotherapeutic agents. Doxorubicin is commonly applied in the treatment of solid tumors, including breast, liver, endometrial, and esophageal cancers, as well as sarcomas and lymphomas, whereas daunorubicin is used predominantly in the treatment of acute myeloid leukemia. [177] Their cytotoxic and cytostatic effects arise from multiple, partially overlapping mechanisms, including DNA intercalation, inhibition of macromolecular biosynthesis, generation of reactive oxygen species, DNA cross-linking, disruption of helicase activity, and interference with topoisomerase II. [177, 178] In addition, the quinone–hydroquinone system present in their structure enables reversible redox cycling, leading to the formation of reactive oxygen species such as superoxide anions and hydrogen peroxide, which contribute to oxidative damage of DNA and cellular membranes. [179]

Structurally, anthracyclines share a conserved framework consisting of a planar tetracyclic hydroxyanthraquinone aglycone linked via a glycosidic bond to an amino sugar, typically daunosamine. [170, 174] This rigid aromatic chromophore enables efficient intercalation between DNA base pairs, while the amino sugar contributes to sequence recognition, electrostatic interactions, and stabilisation within the DNA minor groove. The quinone and hydroquinone functionalities within the aglycone act as electron acceptor and donor groups, respectively, underpinning the redox activity of these molecules. [178] Despite this conserved scaffold, subtle

structural variations among doxorubicin, daunorubicin, idarubicin, and epirubicin give rise to pronounced differences in physicochemical properties, DNA binding behaviour, pharmacokinetics, and toxicity profiles.

The clinically important anthracyclines differ mainly in their aglycone substituents and sugar moieties, which significantly influence intercalation strength, DNA binding specificity, and interactions with cellular targets. [180, 181, 182, 183, 184, 185, 186, 187] For example, epirubicin differs from doxorubicin by inversion of the hydroxyl group at the 4' position of the amino sugar, a stereochemical change that reduces intercalation strength and contributes to its lower cardiotoxicity. [182, 183, 187] Idarubicin lacks a methoxy group at the 4-position of the chromophore, increasing its lipophilicity, enhancing cellular uptake, and enabling oral administration. [184, 185] Although these structural modifications influence DNA binding, differences in pharmacological profiles also reflect additional factors such as membrane transport and chromatin interactions. [170, 171, 175, 184, 188, 189, 190, 191, 192]

Within this family, the relationship between chemical structure and DNA-binding behaviour is central to both biophysical interpretation and pharmacological function. Anthracyclines are DNA-binding anticancer agents whose fluorescence and binding properties are closely linked to their interactions with nucleic acids. Single-molecule and fluorescence-based studies indicate that these compounds share a common two-step binding mechanism involving primary intercalation followed by secondary stacking or groove interactions, with broadly similar equilibrium behaviour and typical binding site sizes of approximately 2–4 base pairs. [188] Such structure–activity relationships underpin both the therapeutic efficacy and the toxicity of anthracyclines and continue to guide the rational design of improved analogues with preserved antitumor activity and reduced adverse effects. [174, 185]

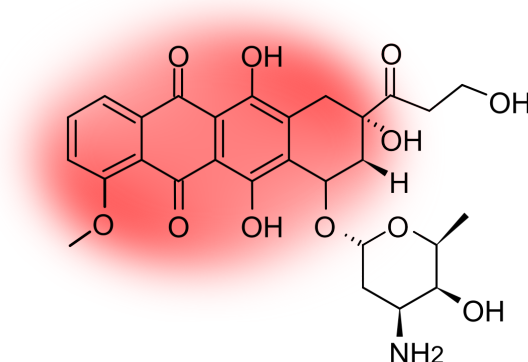


Fig. 11: 2D structure of doxorubicin. The red area marks the aromatic structure responsible for fluorescence.

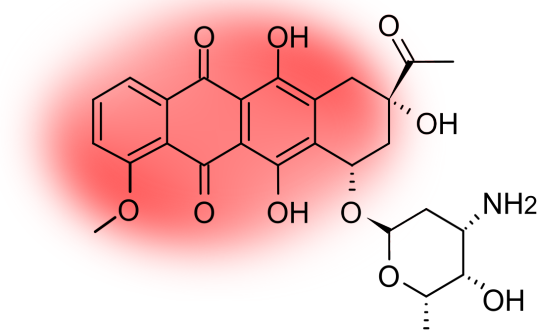


Fig. 12: 2D structure of daunorubicin. The red area marks the aromatic structure responsible for fluorescence.

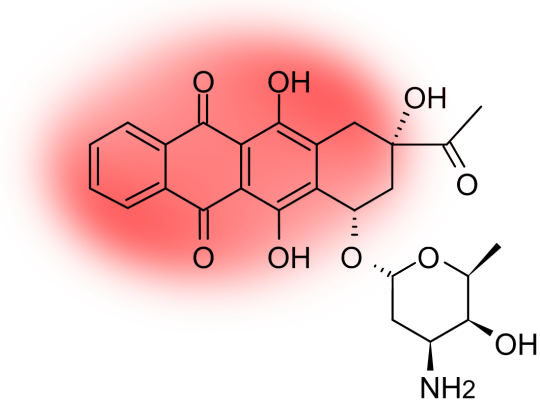


Fig. 13: 2D structure of idarubicin. The red area marks the aromatic structure responsible for fluorescence.

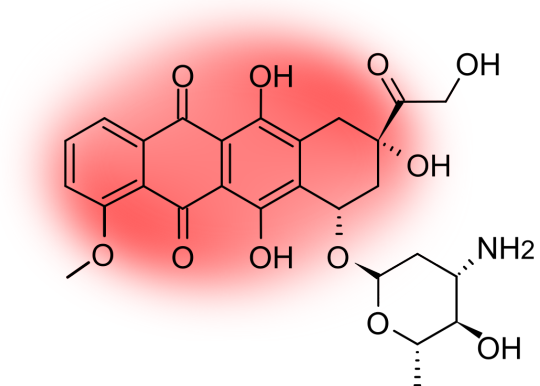


Fig. 14: 2D structure of epirubicin. The red area marks the aromatic structure responsible for fluorescence.

Mechanism of DNA Binding

The principal molecular target of anthracyclines is DNA. Chemotherapy remains a cornerstone of cancer treatment, and many anticancer agents exert their cytotoxic effects through direct interactions with the genetic material. [193] Small molecules interact with DNA primarily through two major binding modes: intercalation and minor groove binding. Intercalators insert a planar aromatic moiety between adjacent base pairs, resulting in elongation and unwinding of the double helix, whereas minor groove binders are typically crescent-shaped molecules that fit into the minor groove with minimal perturbation of the global DNA structure. Among DNA-binding small molecules, anthracycline antibiotics are among the most thoroughly characterised intercalators. [173]

Anthracyclines interact with DNA predominantly through intercalation, although additional noncovalent interactions contribute to overall binding stability and specificity. Intercalation involves insertion of the planar anthraquinone chromophore between adjacent DNA base pairs (Fig. 15), driven primarily by π - π stacking interactions and other non-electrostatic forces. [194, 195, 196, 197] In native B-form DNA, the twist angle between successive base pairs is approximately 36° ; upon anthracycline intercalation, this angle is reduced by 10 – 26° , resulting in local helix unwinding and an increase in DNA contour length of approximately 0.34 nm per intercalated molecule. [175] Although different intercalators vary in the degree of helix unwinding they induce, the increase in contour length is broadly similar across this class of compounds. Thus, intercalation into DNA base pairs represents the primary mechanism of anthracycline–DNA association, and the resulting structural perturbations of the double helix provide the basis for downstream biological effects.

At the molecular level, anthracyclines intercalate through their tetracyclic anthraquinone core, with the B and C rings stacking between adjacent base pairs while the D ring extends through the intercalation site. The attached amino sugar projects into the minor groove, where it participates in hydrogen bonding and electrostatic interactions with DNA bases and the phosphate backbone, contributing significantly to binding free energy. [170, 171, 194, 198] In the biologically relevant context, the therapeutically active species is often a ternary topoisomerase II–DNA–drug complex, in which the intercalated chromophore stabilises the cleavage site while the sugar moiety interacts with both DNA and the enzyme, acting as a “molecular doorstop” that prevents religation of DNA strands. [171, 174, 199, 200]

Although intercalation constitutes the dominant binding mode, anthracyclines do not bind DNA exclusively through this mechanism. Groove contacts, hydrogen bonding, and electrostatic interactions with the phosphate backbone play essential roles in determining binding specificity and stability. [170, 194, 195, 196, 197, 198, 201, 202, 203]

The interaction of anthracyclines with DNA is strongly influenced by sequence composition and local DNA structure. Classical anthracyclines such as doxorubicin and daunorubicin generally exhibit a preference for GC-rich regions, particularly CpG steps, where favourable stacking interactions and specific hydrogen bonding enhance binding affinity. [175, 204, 205, 206, 207] Experimental and computational studies using model oligomers (e.g., d(GCGCGC)₂ versus d(TATATA)₂)

demonstrate that relatively small structural modifications to the drug can shift sequence preference between CG and TA/AT steps by several kilocalories per mole, corresponding to 10–100-fold differences in binding affinity. [208, 209, 210]

Anthracycline–DNA binding is further modulated by solution conditions and DNA topology. Binding affinity depends sensitively on ionic strength, base composition, and whether DNA is present as naked duplex, nucleosomal DNA, or short oligonucleotides. [175, 190, 195, 196, 198, 208, 209, 210, 211, 212] Increasing NaCl concentration reduces binding constants by orders of magnitude for doxorubicin and daunorubicin, reflecting reduced electrostatic attraction and salt-induced conformational changes in DNA. [195, 196, 198, 211] Higher ionic strength can also decrease the apparent number of accessible binding sites, particularly for daunorubicin, consistent with structural changes that mask or alter intercalation sites. [190, 195, 211] Binding affinity is highest under low-salt conditions, in preferred sequence motifs, and for accessible, unwrapped linear DNA, whereas increasing ionic strength, chromatin compaction, or deviation from preferred sequence contexts can reduce effective binding affinity by one or more orders of magnitude.

Collectively, anthracycline binding induces substantial structural perturbations in DNA, including helix unwinding, lengthening, reduced stiffness, and increased susceptibility to strand breakage. [175] These changes interfere with essential processes such as DNA replication and transcription and contribute directly to cytotoxic activity. At the cellular level, doxorubicin accumulates in the nucleus, where intercalation into DNA generates torsional stress, promotes the formation of DNA breaks, and disrupts chromatin organization. Such alterations in DNA topology and chromatin structure are central to the anticancer activity of anthracyclines, although they may also contribute to mutagenesis and long-term adverse effects. [105]

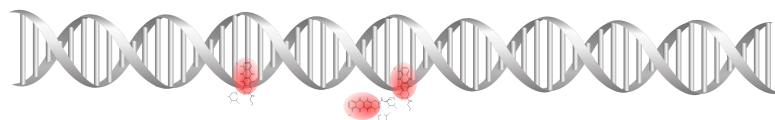


Fig. 15: Scheme of DOX–DNA complex formation. Doxorubicin intercalates into the DNA helix between base pairs. In excess of doxorubicin, the second complex occurs; additional doxorubicin molecules create a complex with the DOX–DNA complex. The mechanism of the reaction is analogous for daunorubicin, idarubicin and epirubicin.

Fluorescence and Photophysics of Anthracyclines

Anthracyclines possess intrinsic fluorescence arising from their conjugated anthraquinone chromophore, and their absorption and emission properties are highly sensitive to the local molecular environment. In aqueous solution, free anthracyclines typically exhibit relatively simple fluorescence decays — often close to mono-exponential for compounds such as daunorubicin — and moderate quantum yields, although solvent interactions, self-quenching, aggregation, and non-radiative decay pathways can limit fluorescence efficiency. [213] Upon DNA binding, however, fluorescence is markedly quenched, the effective quantum yield is reduced, and emission becomes strongly dependent on DNA sequence and binding mode. [173, 213, 214, 215, 216, 217, 218]

DNA-bound anthracyclines exhibit substantially more complex photophysical behaviour than their free counterparts. Fluorescence decays become multi-

exponential, reflecting the coexistence of multiple emissive populations associated with distinct binding configurations, including backbone - or minor-groove-bound states and different intercalative conformations with distinct radiative and non-radiative decay rates. [213]

Intercalation into stacked DNA base pairs is generally associated with strong fluorescence quenching, often approaching near-complete loss of emission at preferred GC-rich or CpG sites. [213, 217, 218] In contrast, weaker intercalative modes or non-intercalative backbone and groove binding retain comparatively higher fluorescence intensity and longer lifetimes. [213, 219] Quantum-yield estimates derived from time-resolved data indicate that backbone- or groove-bound species contribute disproportionately to the observed fluorescence, whereas intercalated species are much more strongly quenched. [213]

Several mechanisms underlie the pronounced quenching observed upon DNA binding. Charge-transfer and electron-transfer interactions between the anthracycline chromophore and guanine bases are considered central contributors, particularly at intercalation sites. [217, 218] In addition, excitonic or energy-transfer interactions between closely spaced bound molecules can further enhance non-radiative decay.

Additional complexity arises from anthracycline self-association in solution. These compounds are known to form dimers and higher-order aggregates, which are typically weakly fluorescent or non-fluorescent. [220, 221, 222, 223, 224, 225] Doxorubicin, in particular, can form supramolecular aggregates or chain-like assemblies at elevated concentrations and ionic strengths. [222] Such self-association can significantly distort fluorescence-based measurements, including fluorescence correlation spectroscopy (FCS), if not properly accounted for.

The presence of multiple emissive and non-emissive states has important implications for fluorescence-based analyses. In FCS, molecular brightness reflects the number of photons emitted per molecule and therefore depends not only on molecular concentration but also on the photophysical properties of each species. Because anthracycline–DNA systems involve a mixture of free drug, groove-bound, intercalated, and potentially aggregated states, the observed brightness represents a population-weighted average over these species rather than a single intrinsic value. [213, 218] Furthermore, DNA binding introduces additional photophysical phenomena, including altered lifetimes, blinking-like behaviour, and increased photobleaching rates, all of which can bias quantitative interpretation if not explicitly modelled. [188, 213, 226, 227, 228]

Consequently, single-molecule brightness analysis requires careful calibration of species-specific brightness values for free and bound states. Differences in fluorescence lifetime, non-radiative decay rates, and photostability can lead to systematic underestimation of bound fractions if uniform brightness is assumed. [188, 213] Moreover, the coexistence of multiple bound states with distinct photophysical properties broadens the brightness distribution and limits the applicability of simple two-state models. [188, 227, 228] These considerations highlight the critical importance of photophysical heterogeneity in interpreting fluorescence data and underscore the need for integrated approaches when using fluorescence-based methods to study anthracycline–DNA interactions.

Equilibrium Constants and Thermodynamics of Binding

The equilibrium association constant (K) of anthracycline–DNA binding provides a quantitative measure of complex stability and offers critical insight into how binding responds to changes in physicochemical conditions. Fluorescence-based techniques are particularly well suited for determining equilibrium constants, as they enable measurements at subnanomolar concentrations and in very small sample volumes while remaining sensitive to changes in binding mode and environment. [229]

Anthracycline–DNA binding is typically very tight, with reported equilibrium association constants spanning approximately 10^4 to 10^8 M^{-1} , depending on the specific drug, DNA sequence, binding mode, and experimental conditions. [176, 188, 192, 220] For the classical anthracyclines daunorubicin and doxorubicin, macroscopic binding constants of $\sim 10^5$ – 10^6 M^{-1} are commonly reported at moderate ionic strength (approximately 0.1–0.2 M salt), whereas low-salt conditions or specific mixed groove–intercalation states can increase binding to $\sim 10^8$ M^{-1} . [176, 192, 220] Literature compilations and single-molecule brightness analyses place most anthracycline–DNA association constants in the 10^4 – 10^8 M^{-1} range, helping to explain the historical scatter of reported values obtained under differing experimental conditions. [188]

The extent and stability of anthracycline intercalation are not fixed properties but depend strongly on molecular context and experimental conditions. This is biologically significant because studies involving various anthracyclines suggest that stronger and more persistent intercalation is closely linked to the ability of these agents to form and stabilise cleavable topoisomerase II–DNA complexes. [176] For doxorubicin in particular, two major DNA-bound states have been described. At low drug loading, when $C(\text{DOX})/C(\text{DNA}) < 0.3$, a high-affinity monomeric or bifunctional DOX–DNA complex predominates, with $K_1 \approx 2.3 \times 10^8$ M^{-1} . At higher drug loading, a second, weaker complex forms in which an additional doxorubicin molecule associates externally with the pre-existing DOX–DNA complex, yielding an “external aggregate” state with $K_2 \approx 9.3 \times 10^5$ M^{-1} . [176] These observations underscore that anthracycline binding cannot always be described by a single uniform equilibrium but may instead involve multiple, nonequivalent binding states.

The dependence of the binding equilibrium on ionic strength is one of the most consistent and dominant features observed across experimental studies. Anthracycline–DNA affinity is strongly modulated by ionic strength, base composition, and whether DNA is present as naked duplex, nucleosomal DNA, or short oligonucleotides. [175, 190, 195, 196, 198, 208, 209, 210, 211, 212] Increasing NaCl concentration reduces binding constants by orders of magnitude for doxorubicin and daunorubicin, reflecting both diminished electrostatic attraction between the positively charged drug and the negatively charged DNA backbone and salt-induced DNA conformational changes. [195, 196, 198, 211] Elevated ionic strength can also decrease the apparent number of available binding sites, particularly for daunorubicin, consistent with salt-driven masking or alteration of intercalation sites arising from changes in DNA structure. [190, 195, 211] Thermodynamic decomposition of binding free energy reveals a substantial polyelectrolyte, or ion-release, contribution, such that the equilibrium association constant decreases approxi-

mately linearly with $\log[\text{Na}^+]$. [196, 198, 211] These findings demonstrate that electrostatic interactions play a central role in determining the thermodynamics of anthracycline–DNA association.

Beyond ionic strength, binding affinity is further modulated by DNA sequence composition, topology, and accessibility. Anthracycline binding is strongest under low-salt conditions, in preferred sequence motifs—often GC-rich regions or specific AT-containing sites—and for accessible, unwrapped linear DNA substrates. In contrast, increasing ionic strength, packaging of DNA into nucleosomes, or deviation from preferred sequence motifs can each reduce effective anthracycline–DNA affinity by one or more orders of magnitude. [175, 190, 195, 196, 198, 208, 209, 210, 211, 212] Free anthracyclines may also self-associate in solution, predominantly as dimers or higher aggregates, but the corresponding self-association constants are typically far smaller than DNA-binding constants; for example, the dimerization constant of daunorubicin is on the order of 10^3 M^{-1} , well below the affinity observed for DNA binding. [176, 198, 220, 221] Thus, under most relevant conditions, DNA association is thermodynamically favoured over self-association, although aggregation can still complicate experimental interpretation at high drug concentration.

Anthracycline–DNA binding is often not purely independent but positively cooperative along the DNA lattice. [188, 230, 231] Temperature-jump kinetic studies of daunorubicin and doxorubicin are well described by a single-step cooperative binding model with nearest-neighbour interactions and overlapping binding sites. [230] In this framework, adjacent bound ligands stabilise one another; notably, doxorubicin dissociates approximately nine times more frequently from an isolated site than from a site flanked by two neighbouring drug molecules, providing a clear signature of positive cooperativity. [230] These results imply that once an anthracycline intercalates, binding at nearby sites becomes more favourable rather than remaining independent. [230] This view is further supported by dual-mode models for doxorubicin binding, in which a strong primary intercalative binding event is followed by a weaker secondary stacking or external binding reaction on drug-decorated DNA. [188] Some disaccharide anthracycline analogues can even bridge or contact two DNA duplexes simultaneously, introducing further coupling between binding events across DNA strands. [231] Across both classical and modified anthracyclines, binding along the DNA helix is therefore more accurately described by cooperative models with overlapping sites and nearest-neighbour effects than by models based on independent, identical binding sites.

Collectively, thermodynamic analyses indicate that anthracycline intercalation and associated binding modes are governed by a balance of favourable enthalpic contributions, including π – π stacking and hydrogen bonding, and entropic factors arising from solvent and counterion release as well as DNA deformation. At the same time, the overall equilibrium behaviour reflects the interplay of electrostatic screening, sequence selectivity, DNA accessibility, ligand self-association, and cooperative nearest-neighbour interactions. Together, these factors determine the experimentally observed equilibrium constants and explain why anthracycline–DNA binding is best understood as a heterogeneous and context-dependent thermodynamic process rather than a single uniform binding event.

Stoichiometry and Saturation of Binding

Structural constraints imposed by intercalation limit the maximum loading of anthracyclines on DNA. Each intercalation event requires local separation of adjacent base pairs and introduces steric and conformational strain into the double helix, thereby restricting the number of molecules that can bind per unit length of DNA. The strength and heterogeneity of anthracycline–DNA interactions therefore make binding stoichiometry and saturation behaviour central parameters for quantitative interpretation, particularly in fluorescence-based measurements where distinct bound states may differ substantially in emission efficiency.

Anthracycline binding cannot be described as a simple one-to-one association with a set of independent, identical DNA sites. The presence of overlapping binding sites, nearest-neighbour interactions, and positive cooperativity means that both the effective number of accessible sites and their occupancy depend on the local binding environment. [188, 230, 231] In addition, higher ionic strength can reduce the apparent number of available binding sites, particularly for daunorubicin, consistent with salt-induced DNA structural changes that mask or alter intercalation sites. [190, 195, 211] Saturation behaviour is therefore context-dependent and influenced by DNA sequence, topology, and solution conditions.

For classical monomeric anthracyclines, neighbour-exclusion models indicate that each bound molecule occupies approximately 3–4 base pairs. [211, 220] Fits to binding data yield an exclusion parameter of approximately 3.1–3.3 base pairs per bound drug, corresponding to a maximum loading of roughly 0.30–0.33 drug molecules per base pair on naked DNA. [211, 220] Thus, saturation is approached at about one anthracycline molecule per 3–4 base pairs, with further loading prevented by steric clash and severe distortion of the DNA helix. Bis-anthracyclines can span a larger segment, up to approximately 6 base pairs per molecule, while still obeying related exclusion constraints. [211, 220] These structural limitations define the upper bound of occupancy even before additional effects of sequence preference or chromatin organisation are considered.

DNA binding also alters the aggregation state of anthracyclines in solution. Because DNA-binding constants are much larger than self-association constants, DNA acts as a strong sink for monomeric drug, shifting the equilibrium away from free aggregates in solution and toward DNA-bound complexes. [176, 188, 198, 220, 221] At low drug-to-DNA ratios, most anthracycline is present as tightly bound monomeric complexes, often accompanied by strong fluorescence quenching and altered brightness relative to the free molecule. [176, 188] At higher loading, additional external or stacked drug–DNA aggregates may form, as observed for doxorubicin, producing lower-brightness states and possible excitonic quenching. [176, 188] Saturation should therefore be understood not merely as filling of identical intercalation sites, but as a transition through multiple occupancy regimes with distinct structural and photophysical properties.

Fluorescence correlation spectroscopy (FCS) provides a powerful framework for probing these binding equilibria, particularly for distinguishing free anthracycline from DNA-bound species. In label-free FCS studies of doxorubicin, autocorrelation curves were successfully described using two diffusing components corresponding to free drug and more slowly diffusing DOX–DNA complexes. [232] This allows robust discrimination between unbound and DNA-associated anth-

racycline. However, when multiple drug molecules bind to the same DNA chain, the resulting complexes often have very similar diffusion coefficients, so conventional FCS detects them as a single, slowly diffusing population rather than as individually resolved stoichiometric states. [232, 233]

For this reason, separating singly bound from multiply bound DNA complexes generally requires more than diffusion analysis alone. Theory and methodological studies show that conventional single-colour FCS can resolve distinct species only when they differ sufficiently in diffusion coefficient or molecular brightness; otherwise, they are not uniquely identifiable from the autocorrelation function. [153, 234, 235] Single-molecule brightness analysis has therefore been used to complement FCS and to identify multiple binding reactions with distinct emissive signatures. [188, 236] In anthracycline systems, combining FCS with brightness or burst analysis can partially separate low-occupancy and high-occupancy DNA complexes, even when their diffusion properties are similar. [188, 236]

Taken together, these observations show that anthracycline saturation on DNA is governed by neighbour exclusion, cooperative effects, DNA structural context, and the emergence of higher-order drug–DNA states at elevated loading. FCS robustly distinguishes free from DNA-bound anthracycline, but discrimination between singly and multiply occupied DNA complexes is not generally achievable from standard diffusion-based analysis alone. Such resolution becomes feasible only to a limited extent when FCS is coupled to molecular brightness or related single-molecule observables and is interpreted using appropriate multi-site binding models. [153, 188, 232, 233, 234, 235, 236]

Kinetics of Binding and Dissociation

Anthracycline binding to DNA is a dynamic, multistep process characterised by association and dissociation events over a range of timescales. Intercalation is not an instantaneous event but may proceed through transient intermediate states, including backbone- or minor-groove-bound conformations and partially inserted structures, before reaching a fully intercalated configuration. [213] This structural heterogeneity implies that binding and dissociation cannot be described by a single uniform pathway but instead involve multiple conformational states with distinct kinetic and photophysical properties.

Kinetic studies indicate that anthracyclines bind DNA with relatively similar association rate constants, typically on the order of 10^6 – 10^7 $\text{M}^{-1} \text{s}^{-1}$, but exhibit markedly different dissociation rates, ranging from approximately 3 to 35 s^{-1} . [176, 188, 198, 230, 237] As a result, differences in binding affinity among related anthracyclines arise primarily from variations in dissociation kinetics rather than in the initial encounter rate. In this sense, the “stickiness” of anthracycline–DNA complexes is governed predominantly by residence time on DNA, which reflects the stability of the intercalated or mixed binding state.

Temperature-jump kinetic experiments for daunorubicin and doxorubicin are well described by a cooperative binding model that incorporates nearest-neighbour interactions and overlapping binding sites along the DNA lattice. [230] Within this framework, dissociation rates depend strongly on the local binding environment. For example, doxorubicin molecules dissociate approximately nine times more rapidly from an isolated site than from a site flanked by two neighbouring bound ligands, providing clear evidence of positive cooperativity. [230] These results

demonstrate that binding kinetics are not uniform but are modulated by local occupancy and intermolecular interactions, such that adjacent bound molecules stabilise one another and effectively prolong residence time.

This cooperative behaviour is further consistent with models invoking multiple, non-equivalent binding states. In particular, dual-mode binding has been proposed for doxorubicin, involving a strong primary intercalative interaction followed by weaker secondary stacking or external binding on drug-decorated DNA. [188] Such models imply that binding and dissociation proceed through a hierarchy of states rather than through a single reversible step, and that local ligand density influences both the probability of binding and the lifetime of bound states.

From a methodological perspective, these kinetic features have important implications for fluorescence correlation spectroscopy (FCS) and related fluctuation-based techniques. In FCS, free anthracycline and DNA-bound complexes can often be distinguished through their differing diffusion times. However, the presence of multiple bound states with similar diffusional properties means that fast interconversion between states and transient conformations cannot be resolved solely through diffusion analysis. [153, 188, 232, 233, 234, 235, 236] Instead, accurate interpretation requires reaction-aware models that account for interconversion kinetics on the timescale of observation.

In systems where binding and unbinding occur on timescales comparable to diffusion through the observation volume, the measured signal reflects a dynamic equilibrium rather than a static mixture of species. Under such conditions, additional observables, such as molecular brightness or burst-resolved analysis, are often required to distinguish between different binding modes and occupancies. [153, 188, 232, 233, 234, 235, 236] Thus, while FCS provides a powerful tool for probing anthracycline–DNA interactions, resolving the full complexity of binding kinetics requires integration of diffusion, photophysical, and kinetic information within appropriate multi-state models.

Collectively, these observations indicate that anthracycline–DNA binding is governed by a combination of intrinsic molecular properties and cooperative interactions along the DNA lattice, resulting in kinetic behaviour that depends on local occupancy, binding mode, and sequence context.

Self-Association and Aggregation of Anthracyclines

Anthracyclines are prone to self-association and aggregation in aqueous environments, particularly at elevated concentrations. These processes arise from π – π stacking interactions between the planar aromatic chromophores and can significantly alter photophysical behaviour, typically resulting in strong fluorescence quenching and reduced apparent molecular brightness. Such self-association competes with DNA binding, introducing additional complexity into the interpretation of fluorescence-based measurements.

Anthracycline self-association most commonly leads to the formation of dimers and higher-order aggregates that are weakly fluorescent or effectively non-emissive. [220, 221, 222, 223, 224, 225] For daunorubicin, aggregation becomes significant above approximately 10 μ M and can be described by an indefinite, stepwise stacking model with an intrinsic association constant on the order of $1.5 \times 10^3 \text{ M}^{-1}$. [220] Doxorubicin similarly undergoes dimerisation, but at higher concentrations and ionic strengths, it can further assemble into supramolecular chains or gel-like

structures; in such assemblies, dimers are essentially non-fluorescent. [222] More generally, self-association has been confirmed across anthracycline analogues, including doxorubicin, daunorubicin, and epirubicin, using spectroscopic and structural methods. [221]

In typical biophysical experiments conducted at low micromolar or nanomolar concentrations, the fraction of aggregated species may be small and, in some cases, undetectable by spectroscopy. [220] Nevertheless, even a minor population of non-emissive or weakly emissive aggregates can bias fluorescence measurements. Because such species contribute to molecular mass but not proportionally to fluorescence intensity, they reduce the apparent brightness per molecule and lead to underestimation of the number of emitting species if the system is analysed as a single homogeneous population. [222, 223] Theoretical treatments of fluorescence correlation spectroscopy (FCS) show that autocorrelation amplitudes are weighted by molecular brightness, so mixtures of species with different emissivities can distort both inferred particle numbers and brightness values unless explicitly modelled. [223, 224, 225]

In addition to free-solution aggregation, intermolecular interactions can also occur in the DNA-bound state. The strong fluorescence quenching observed for intercalated anthracyclines has been attributed not only to charge-transfer interactions with guanine bases but also to excitonic or energy-transfer interactions between closely spaced bound chromophores. [217, 218] Dual-mode binding models, in which primary intercalation is followed by secondary stacking or external association on drug-decorated DNA, further support the existence of intermolecular interactions among bound anthracycline molecules. [188] In some cases, disaccharide anthracycline analogues can even bridge or contact two DNA duplexes simultaneously, introducing additional coupling between binding events across DNA strands. [231] These observations indicate that aggregation-like behaviour is not limited to free solution but can also arise within DNA-bound complexes.

The interplay between self-association and DNA binding is governed by their relative thermodynamic strengths. Anthracycline–DNA binding constants (typically 10^6 – 10^8 M⁻¹) exceed self-association constants by several orders of magnitude, so DNA effectively acts as a strong sink for monomeric drug. [176, 198, 220, 221] As a result, at low drug-to-DNA ratios, equilibrium shifts toward monomeric DNA-bound complexes, largely depleting free aggregates in solution. [176, 188, 198] At higher drug loading, however, additional externally associated or stacked drug–DNA aggregates may form, creating new heterogeneous states with distinct structural and photophysical properties. [176, 188]

These features have direct implications for fluorescence correlation spectroscopy and related techniques. In FCS, aggregation manifests as changes in both diffusion coefficients and brightness distributions. Because non-emissive aggregates contribute to mass but not to fluorescence signal, they can alter the apparent number of particles and bias brightness-based quantification. [223, 224, 225] Even at low bulk concentrations, such species may be detectable through changes in autocorrelation amplitude or effective particle number. [223] Consequently, accurate interpretation of FCS data requires strategies to minimise or account for aggregation, including working at appropriately low concentrations, optimising buffer conditions, and incorporating multi-species models or complementary spectroscopy.

pic measurements.

Overall, anthracyclines form stacked, largely non-fluorescent aggregates in aqueous solution at micromolar concentrations and above, and these processes can significantly influence both fluorescence properties and apparent binding behaviour. In systems where DNA is present, the observed signal reflects a balance between self-association and DNA association, as well as the formation of higher-order drug–DNA complexes. Careful consideration of these competing equilibria is therefore essential for reliable quantitative analysis of anthracycline–DNA interactions.

Photostability and DNA-Bound Photophysics

Photobleaching and fluorescence dynamics differ markedly between free and DNA-bound anthracyclines, reflecting substantial changes in their photophysical behaviour upon interaction with nucleic acids. DNA binding profoundly alters anthracycline emission, producing heterogeneous states that are often strongly quenched, with reduced effective quantum yields and a pronounced dependence on sequence and binding mode. [173, 213, 214, 215, 216, 217, 218] These observations indicate that the photophysics of DNA-bound anthracyclines must be considered separately from those of the free dye, as binding introduces new emissive states, decay pathways, and environmental constraints.

At the molecular level, anthracycline photophysics is closely linked to their redox-active quinone–hydroquinone system. These compounds can act as electron acceptors in enzymatically or chemically driven redox reactions, forming semiquinone radical intermediates that participate in the generation of reactive oxygen species such as superoxide anions, hydroxyl radicals, and peroxides. [177] While these processes are central to the biological activity and toxicity of anthracyclines, they also have important photophysical consequences. In particular, electron-transfer and radical-generating pathways contribute to fluorescence quenching and to the modulation of molecular brightness under illumination.

DNA binding modifies these processes in a complex manner. On one hand, association with DNA can reduce free-drug photodegradation and suppress bulk photo-redox activity in solution. [207, 238, 239] For several anthraquinone-based drugs, increasing the DNA base-pair-to-drug ratio significantly decreases oxygen consumption and NADH oxidation during illumination, indicating that an intercalated or DNA-bound drug is less active as a photosensitizer in bulk solution. [238] This behaviour suggests that DNA binding stabilises the chromophore by shifting reactive electronic states into DNA-coupled charge-transfer configurations rather than free-solution photochemistry. [207]

On the other hand, DNA binding does not eliminate photochemical reactivity but instead redistributes it into the local DNA environment. Ultrafast spectroscopic studies of daunorubicin and doxorubicin complexes have shown that excitation can induce rapid charge separation between the drug and DNA bases, followed by redox cycling involving molecular oxygen. [207] In this context, DNA-bound anthracyclines can act as localised photosensitizers, generating reactive oxygen species in close proximity to DNA and thereby promoting strand damage. Similar behaviour has been observed for related intercalators that induce DNA strand breaks under illumination in the presence of electron donors such as NADH. [239] Thus, DNA binding simultaneously suppresses bulk photochemistry while ena-

bling localised, DNA-coupled photochemical pathways.

In addition to these redox effects, DNA binding introduces significant changes in fluorescence lifetime, blinking behaviour, and photostability. Bound anthracyclines exhibit multiple emissive states with distinct lifetimes; for example, daunorubicin displays at least three lifetime components corresponding to different binding modes, while doxorubicin lifetimes vary with DNA microenvironment and damage state. [213, 227] Intercalation into nuclear DNA is associated with strong fluorescence quenching, whereas binding to other cellular components has a much smaller effect. [226] At high local concentrations, excitonic coupling between closely spaced chromophores can further enhance non-radiative decay and accelerate photobleaching. [228] Consequently, DNA-bound anthracyclines may exhibit either enhanced apparent photostability in bulk solution or increased susceptibility to localised photochemical processes, depending on the specific context.

These photophysical changes have direct implications for fluorescence-based measurements. DNA binding creates new lifetime distributions and blinking-like dynamics that can introduce additional fluctuations into fluorescence signals. If bound species exhibit shorter lifetimes, higher non-radiative decay rates, or faster photobleaching, their per-molecule brightness is reduced relative to free drug. As a result, analyses based solely on fluorescence intensity or brightness may underestimate the fraction of DNA-bound anthracycline unless species-specific properties are considered. [188, 213]

For quantitative applications such as fluorescence correlation spectroscopy, these considerations necessitate careful experimental design and data interpretation. Brightness-based methods remain powerful but must incorporate distinct lifetimes, emissivities, and photobleaching behaviours of free and DNA-bound species. In practice, this often requires calibration of molecular brightness across different binding regimes and restricting analysis to early acquisition times to minimise bleaching artefacts. [188, 227] Overall, the interplay between photostability, redox activity, and DNA-coupled photophysics underscores the complexity of anthracycline fluorescence and highlights the need to account explicitly for these effects in quantitative studies of anthracycline–DNA interactions.

Biological and Pharmacological Context

Anthracycline–DNA binding equilibria form a central part of the molecular basis of anthracycline activity in cancer therapy. Many anticancer agents used in clinical practice, including doxorubicin, exert their cytotoxic effects by targeting processes that regulate DNA topology and integrity, most notably those mediated by topoisomerases. [3] Doxorubicin (DOX), a prominent member of the anthracycline family, remains one of the most effective anticancer agents in clinical use. Since its discovery more than 5 decades ago, it has attracted sustained research interest; however, despite extensive investigation, its precise molecular mechanism of action remains unresolved. The prevailing view is that DOX acts primarily by inhibiting human type IIA topoisomerase, both by stabilising the cleavage complex and by catalytic inhibition, while its characteristic modes of DNA binding play a central role in determining its biological effects. [176]

Anthracyclines intercalate into DNA by inserting their planar tetracyclic chromophore between adjacent base pairs, thereby inducing substantial distortions in the polynucleotide structure and interfering with essential processes such as

DNA replication and transcription. [176] These distortions are not merely secondary consequences of binding but are directly connected to pharmacological function, because the extent and stability of intercalation influence the formation and persistence of cleavable topoisomerase II–DNA complexes. Anthracyclines, therefore, act primarily as topoisomerase II poisons, converting this essential enzyme into a source of persistent DNA double-strand breaks that ultimately trigger apoptosis. [169, 172, 240] The therapeutically relevant lesion is a ternary anthracycline–DNA–topoisomerase II complex in which religation of cleaved DNA is blocked, stabilising the cleavage complex and transforming topoisomerase II into a potent cytotoxic mediator. [171, 172, 179]

DNA binding by anthracyclines is strongly sequence- and context-dependent, and these properties are directly relevant to their biological activity. Binding affinity is highest in preferred sequence motifs, often GC-rich regions, under low-salt conditions and for accessible DNA structures, whereas chromatin compaction, salt concentration, and local sequence composition modulate both the extent of binding and the stability of drug–DNA complexes. [175, 190, 195, 196, 198, 208, 209, 210, 211, 212] Cooperative interactions along the DNA lattice, together with dissociation-controlled residence times, further shape the persistence of anthracycline binding and thus influence the extent of topoisomerase poisoning, DNA damage induction, and disruption of DNA-dependent processes. In this sense, pharmacological activity depends not simply on how tightly anthracyclines bind DNA, but on how and where they bind, how long they remain associated, and what structural consequences result.

Doxorubicin and daunorubicin have been used for decades in the treatment of a broad spectrum of malignancies. Doxorubicin is widely employed against solid tumours, including breast, biliary, endometrial, oesophageal, and liver cancers, as well as osteosarcomas, soft-tissue sarcomas, and non-Hodgkin’s lymphoma, whereas daunorubicin is used predominantly in the treatment of acute myeloid leukaemia. [177] More broadly, anthracyclines remain among the most important agents in cancer chemotherapy. [173] Their clinical value reflects not only potent cytotoxicity in rapidly proliferating cells but also the fact that they are incorporated into multiple curative or disease-controlling regimens across hematologic and solid malignancies. [172]

Although topoisomerase II poisoning is regarded as the classical and dominant mechanism of anthracycline action, these compounds exert pleiotropic effects on cellular physiology. Multiple mechanisms have been proposed to account for their cytostatic and cytotoxic activity, including inhibition of macromolecular biosynthesis through DNA intercalation, direct interference with DNA and RNA synthesis, generation of reactive oxygen species, lipid peroxidation, formation of covalent DNA adducts and cross-links, disruption of helicase activity and DNA unwinding, membrane perturbation, and direct chromatin damage. [177, 178, 179, 189] Early studies emphasised suppression of DNA biosynthesis, likely arising from a combination of intercalation and direct interference with DNA polymerases. [177] More recent work has highlighted that intercalation-driven chromatin damage, including nucleosome destabilisation and histone eviction, makes a substantial contribution to anthracycline efficacy and may help explain biological effects not captured by topoisomerase poisoning alone. [105, 171, 189]

Reactive oxygen species and redox chemistry also contribute to anthracycline pharmacology and toxicity, although their precise role in antitumor action remains complex and context-dependent. The quinone moiety of doxorubicin and daunorubicin can accept electrons in enzyme-catalysed redox reactions, producing semiquinone radicals that either directly damage DNA or react with oxygen to generate superoxide, hydroxyl radicals, and peroxides. [177, 179] These pathways are widely implicated in oxidative stress, lipid peroxidation, mitochondrial dysfunction, and protein-associated DNA damage. [174, 177, 179, 241] However, ROS generation is generally regarded as a contributor to anthracycline-induced toxicity — especially cardiotoxicity — rather than the primary mechanism underlying anticancer efficacy. [174, 177, 241]

Anthracycline-induced cardiotoxicity remains the major dose-limiting adverse effect of this drug class. Clinically, it may manifest as cardiomyopathy, ventricular dysfunction, arrhythmias, or pericarditis–myocarditis syndrome, and is typically cumulative and often irreversible. [171, 179] Doxorubicin therapy is therefore commonly limited to cumulative doses of approximately $450\text{--}550\text{ mg} \cdot \text{m}^{-2}$ in order to reduce the risk of severe cardiac injury. [171] Cardiac susceptibility is thought to arise from the high mitochondrial content of cardiomyocytes, their limited antioxidant capacity, and the ability of anthracyclines to undergo redox cycling and iron-mediated radical formation. [105, 171, 179] Complexes involving iron and anthracyclines can enhance hydroxyl radical generation and lipid peroxidation, further contributing to membrane damage and mitochondrial dysfunction. [179, 191] In addition to cardiotoxicity, anthracycline therapy is associated with myelosuppression, mucosal and epithelial toxicity, gonadotoxicity, and therapy-related secondary malignancies, reflecting the lack of selectivity for malignant over normal proliferating tissues. [105, 171]

Anthracyclines can also induce DNA damage through mechanisms other than noncovalent intercalation and topoisomerase poisoning. Under certain conditions, these drugs can form covalent DNA adducts or cross-links, often via formaldehyde-mediated Schiff base intermediates involving guanine residues. [177, 179, 242, 243] Such lesions can block replication and transcription more strongly than simple intercalation and may enhance cytotoxicity. At the same time, site selectivity on DNA, selectivity between topoisomerase II isoforms, and the balance between DNA damage and chromatin damage all influence the biological outcome. [172, 189, 244] In particular, inhibition of Top2 α is generally regarded as the main source of antitumor efficacy, whereas Top2 β is implicated more strongly in long-term toxicities, including cardiotoxicity and therapy-related secondary cancers. [244]

Differences among doxorubicin, daunorubicin, epirubicin, and idarubicin illustrate that DNA binding is only one component of their pharmacological profile. Although these compounds share broadly similar equilibrium behaviour and a common two-step binding mechanism involving intercalation followed by secondary stacking or groove-associated interactions, their clinical properties differ substantially. [188] These distinctions reflect not only subtle differences in DNA-binding strength, kinetics, and local DNA perturbation, but also differences in membrane transport, lipophilicity, cellular uptake, iron-mediated chemistry, and chromatin effects. [170, 171, 175, 184, 188, 189, 190, 191, 192] For example, ida-

rubicin is more lipophilic and accumulates more efficiently in drug-sensitive and multidrug-resistant cells, while epirubicin perturbs DNA less strongly and is associated with lower cardiotoxicity. [175, 184] Thus, variations in DNA-binding behaviour align only partly with pharmacological differences; membrane handling, chromatin-level damage, and redox reactivity are also crucial determinants of clinical outcome.

Overall, anthracycline pharmacology emerges from the interplay of DNA intercalation, topoisomerase II poisoning, sequence- and context-dependent residence on DNA, ROS generation, covalent adduct formation, and chromatin disruption. Binding equilibria matter because they govern not only whether anthracyclines associate with DNA, but also the persistence, location, and structural consequences of that association. In turn, these factors determine formation and stability of topoisomerase cleavage complexes, induction of DNA and chromatin damage, and the balance between therapeutic efficacy and toxicity. A detailed understanding of anthracycline–DNA interactions therefore provides a critical framework for interpreting both the anticancer activity and the adverse effects of this important class of chemotherapeutic agents.

Methodological Considerations for FCS Studies

Molecular brightness analysis by fluorescence correlation spectroscopy (FCS) offers distinct advantages for studying anthracycline–DNA interactions because it relies on a single intrinsic fluorophore and can sensitively report changes in binding state even when diffusion coefficients differ only modestly. This is particularly valuable in anthracycline systems, where DNA binding is typically very tight and highly heterogeneous. Reported anthracycline–DNA equilibrium association constants span approximately 10^5 – 10^{11} M^{−1} depending on the drug, DNA sequence, and binding mode, while most common anthracycline–DNA interactions fall in the 10^4 – 10^8 M^{−1} range under experimentally relevant conditions. [176, 188, 192, 220] These values are strongly modulated by ionic strength, DNA accessibility, sequence context, and ligand structure, which together account for much of the historical scatter in reported binding constants. [175, 190, 195, 196, 198, 208, 209, 210, 211, 212]

At the same time, the fluorescence response of anthracyclines is itself strongly dependent on DNA binding mode and sequence context, which has direct methodological implications for fluorescence-based analysis. DNA binding markedly quenches anthracycline fluorescence, lowers effective quantum yield, and renders emission highly sequence- and mode-dependent. [173, 213, 214, 215, 216, 217, 218] Free anthracyclines in buffer generally exhibit relatively simple fluorescence decays and moderate quantum yields, whereas DNA-bound states become heterogeneous and multi-exponential. [213] For daunorubicin and related anthracyclines, intercalation often causes strong quenching, in some cases approaching near-complete loss of fluorescence at preferred GC-rich sites, whereas backbone- or groove-bound states remain comparatively brighter. [213, 217, 218] Charge-transfer or electron-transfer interactions with guanine bases, together with excitonic or energy-transfer interactions between bound molecules, are implicated in the pronounced quenching observed at certain intercalation sites. [217, 218]

These properties mean that any fluorescence-correlation-based analysis of anthracycline–DNA interactions must explicitly account for fluorescence heteroge-

neity arising from multiple emissive and strongly quenched states. Because anthracycline–DNA affinity is also highly sensitive to ionic strength, base composition, and DNA topology, quantitative interpretation depends critically on experimental conditions. [175, 190, 195, 196, 198, 208, 209, 210, 211, 212] Increasing NaCl concentration reduces binding constants by orders of magnitude, alters electrostatic interactions, and can change DNA conformation sufficiently to reduce the apparent number of accessible binding sites. [190, 195, 196, 198, 211] In addition, cooperative nearest-neighbour effects and multiple binding modes introduce further heterogeneity that must be considered in any mechanistic analysis. [188, 230, 231] Accurate interpretation of FCS data therefore requires careful control of buffer composition, salt concentration, DNA substrate, and ligand-to-DNA ratio.

In principle, FCS can distinguish free anthracycline from DNA-bound anthracycline through differences in diffusion time, but separating singly bound from multiply bound complexes is substantially more difficult. [153, 188, 232, 233, 234, 235, 236] For doxorubicin, a label-free FCS model has been developed in which multiple DOX molecules bind to a single DNA chain; the corresponding analytical autocorrelation function explicitly includes higher-order complexes and yields an overall equilibrium constant for complex formation. [232] In such measurements, the autocorrelation curves are well described by two diffusing components corresponding to free DOX and slower DOX–DNA complexes. [232] This enables clear discrimination between free drug and DNA-bound drug in general, but not between different occupancies on the same DNA chain, because multiply loaded DNA complexes often have very similar diffusion coefficients. [232, 233]

For this reason, conventional single-colour FCS alone is usually insufficient to resolve individual bound stoichiometries unless the species also differ appreciably in molecular brightness. Theoretical and methodological studies emphasise that distinct species can be uniquely resolved from the autocorrelation function only when their diffusion or brightness differs sufficiently. [153, 234, 235] Single-molecule brightness analysis has therefore been used to complement FCS, revealing at least two binding reactions for anthracyclines and assigning different brightness states to distinct complex types. [188, 236] Combining FCS with brightness analysis can thus partially separate low- and high-occupancy complexes even when diffusion is nearly identical. [188, 236] More broadly, single-molecule brightness/FCS studies suggest that doxorubicin, daunorubicin, epirubicin, and idarubicin share a broadly similar two-step binding mechanism involving primary intercalation followed by secondary stacking or groove-associated binding, with measured site sizes in the usual 2–4 base pair range. [188]

A further methodological complication arises from anthracycline self-association. Anthracyclines self-associate in water to form weakly fluorescent or non-fluorescent dimers and, in the case of doxorubicin, higher aggregates. [220, 221, 222, 223, 224, 225] If present, such species can seriously distort FCS brightness-based interpretation. Non-emissive or weakly emissive aggregates reduce the total fluorescence intensity while still contributing to molecular mass, making the apparent monomer brightness smaller and the inferred particle number lower than the true value if the system is analysed as a single homogeneous population. [222, 223] FCS theory predicts that autocorrelation amplitudes are brightness-weighted in mixtures of species with different emissivities, so inferred particle numbers and brightness

values become biased unless species composition is known or explicitly modelled. [223, 224, 225] Anthracyclines form stacked, largely non-fluorescent aggregates in water at low-micromolar concentrations and above, and such aggregates will bias FCS brightness and concentration estimates unless experimentally minimised or analytically accounted for. [220, 221, 222, 223, 224, 225]

DNA binding complicates this picture further by both suppressing and reshaping aggregation. Because DNA binding constants greatly exceed self-association constants, DNA strongly sequesters monomeric anthracycline into high-affinity DNA-bound complexes, thereby depleting free aggregates in solution. [176, 188, 198, 220, 221] At low drug-to-DNA ratios, most drug exists as monomeric DNA-bound complexes, with strong fluorescence quenching and altered brightness relative to free monomer. [176, 188] At higher loading, however, external or stacked drug-DNA aggregates can appear, as in the (DOX – DNA)–DOX state, producing additional low-brightness populations and possible excitonic quenching. [176, 188] Thus, DNA binding largely disassembles free aggregates while at the same time creating new aggregate-like bound states, making FCS brightness curves intrinsically multi-state and non-trivial to interpret.

Another major challenge is that DNA binding alters not only fluorescence intensity but also lifetime, blinking-like behaviour, and photostability. DNA-bound anthracyclines exhibit new lifetime distributions and often faster bleaching, both of which can bias brightness-based quantification if not modelled explicitly. [188, 213, 226, 227, 228] Free daunorubicin is approximately mono-exponential in buffer, whereas DNA-bound daunorubicin exhibits at least three distinct lifetime components associated with backbone-bound and multiple intercalated conformations. [213] If bound species have shorter lifetimes, higher non-radiative decay rates, or faster bleaching, their per-molecule brightness is reduced, so populations inferred from brightness alone will underestimate the fraction of bound drug unless species-specific molecular brightness values are calibrated. [188, 213] Heterogeneous bound states, including intercalated species and externally stacked aggregates, broaden the brightness distribution further and make simple two-state models inadequate for precise quantification. [188, 227, 228] In practice, brightness-based methods remain highly useful, but they require calibration for each relevant binding regime and are best applied under low-bleach conditions. [188, 227]

Taken together, these considerations define both the advantages and the limitations of molecular brightness analysis for anthracycline-DNA studies. Brightness adds important discriminatory power where diffusion alone is insufficient, particularly for distinguishing free drug from bound populations and for partially resolving low- versus high-occupancy complexes. At the same time, fluorescence heterogeneity, cooperative binding, self-association, DNA-bound external aggregation, lifetime complexity, and bleaching all complicate straightforward interpretation. Robust FCS analysis therefore requires explicit multi-state modelling and, where possible, integration with complementary methods such as absorption titration, isothermal titration calorimetry, stopped-flow kinetics, fluorescence lifetime measurements, and single-molecule imaging. Together, these approaches provide the most reliable framework for quantitatively resolving the equilibrium and dynamic behaviour of anthracycline-DNA interactions under well-defined experimental conditions.

1.4 Fluorescence correlation spectroscopy

Fundamentals of Fluorescence and Molecular Brightness

Fluorescence is the process whereby a molecule absorbs light (photons), is promoted to an excited electronic state, and subsequently returns to the ground state by emitting light of longer wavelength. This phenomenon underpins a wide range of applications in imaging, sensing, and materials science. [245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 258, 259, 260] More broadly, the term photophysics encompasses all physical processes initiated by light absorption that do not alter the chemical identity of a molecule, including fluorescence emission, quenching, and intra- or intermolecular energy transfer. [261] Fluorescence itself is a radiative transition between electronic states of the same multiplicity, most commonly from the first excited singlet state, and is characterised by measurable properties such as spectral distribution, polarisation, intensity, and excited-state lifetime. Because these observables are highly sensitive to the local molecular environment, fluorescence provides a powerful means for probing biomolecular structure and dynamics at both ensemble and single-molecule levels. [261]

The essential photophysical parameters of a fluorophore include absorption, emission, quantum yield, fluorescence lifetime, and photostability. Absorption defines the wavelengths at which photons promote the molecule from the ground state to an excited electronic state. [246, 249] Emission corresponds to photon release as the molecule relaxes back to the ground state and typically occurs at longer wavelengths due to the Stokes shift. [246, 249, 252] The fluorescence quantum yield, defined as the ratio of emitted to absorbed photons, quantifies fluorescence efficiency and depends strongly on molecular structure, environment, and competing non-radiative decay pathways. [246, 248, 250, 252, 253, 254, 259] The fluorescence lifetime reflects the average time spent in the excited state and is governed by the balance between radiative and non-radiative processes. [246, 247, 252, 253, 260] Photostability describes resistance to irreversible photobleaching and is crucial for sustained or high-intensity measurements. [245, 247, 251, 255, 256, 258, 260]

Fluorescence correlation spectroscopy (FCS) provides a powerful framework for probing molecular dynamics by analysing fluorescence intensity fluctuations arising from molecules diffusing into and out of a small, optically defined observation volume. [153, 262] In a typical FCS experiment, a focused laser beam excites fluorescent molecules at low concentration, and individual molecules produce transient bursts of fluorescence as they traverse the detection volume. The duration of these bursts reflects the molecule's residence time, while their intensity reflects its molecular brightness. The resulting time-dependent fluorescence signal is analysed using autocorrelation to extract parameters such as local concentration, diffusion coefficient, hydrodynamic radius, and, in some cases, reaction kinetics. [262] Owing to its high temporal resolution and sensitivity, FCS can access timescales from nanoseconds to seconds and detect processes down to the single-molecule level. [153]

In FCS and related fluctuation techniques, molecular brightness (also called counts per molecule, CPM) is defined as the average number of detected fluorescence photons per molecule per unit time within the observation volume. [158, 224, 255, 263] Practically, brightness is obtained by dividing the mean fluorescence count rate $\langle F \rangle$ by the average number of molecules $\langle N \rangle$, which is derived

from the amplitude of the autocorrelation function. [158, 224, 225, 255, 264, 265, 266, 267, 268, 269, 270, 271] Under fixed experimental conditions — defined by excitation intensity, detection efficiency, background signal, and instrument characteristics — molecular brightness is proportional to the fluorophore’s absorption cross-section and quantum yield, while being reduced by dark-state formation, blinking, or other non-radiative pathways. [158, 224, 225, 255, 263, 272]

Importantly, molecular brightness is not an intrinsic constant but a state-dependent observable. In heterogeneous systems, brightness reflects the time-averaged detected emission per molecule and is influenced by the distribution of emissive and non-emissive states. Processes that promote the fluorophore into dark or quenched states, or that reduce its quantum yield, decrease effective brightness, whereas processes that shorten dark-state or triplet lifetimes can increase it. [261, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282] Consequently, in samples containing multiple species or binding states, more advanced analytical approaches — such as photon counting histogram (PCH) analysis, fluorescence intensity distribution analysis (FIDA), or global fitting procedures — are required to resolve distinct brightness populations. [153, 158, 224, 264, 265, 266, 267, 268, 269, 270, 271]

This state dependence underlies the utility of molecular brightness as a reporter of molecular interactions. Changes in local environment, conformational state, or binding — such as association with DNA — can alter excitation efficiency, non-radiative decay rates, and the population of dark states, thereby modulating the detected photon emission rate per molecule. As a result, molecular brightness provides a sensitive, quantitative probe of biomolecular interactions and dynamics, particularly in systems where traditional diffusion-based discrimination is insufficient.

Principles of Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) is a single-molecule sensitive technique that analyses temporal fluctuations in fluorescence intensity arising from fluorescent molecules diffusing through a tightly confined observation volume, typically on the order of a femtoliter or less. [60, 153, 235, 283, 284, 285] These fluctuations originate from stochastic processes driven by thermal motion, including translational diffusion, molecular interactions, conformational changes, and transitions between states with different fluorescence yields. [284, 286, 287]

In a standard confocal implementation, a focused laser beam defines the excitation volume, while a high-numerical-aperture objective collects fluorescence from the same region. The emitted light is spectrally filtered, passed through a confocal pinhole to reject out-of-focus fluorescence, and detected by sensitive single-photon detectors such as avalanche photodiodes. [153, 235, 285] This optical confinement improves the signal-to-noise ratio and enables measurements at very low molecular concentrations. As fluorescent molecules diffuse into and out of the observation volume, they generate bursts of photons whose duration reflects residence time and whose intensity reflects molecular brightness. [153, 262]

The primary observable in FCS is the time-dependent fluorescence intensity, $F(t)$, which fluctuates around its mean value. These fluctuations are analysed using the temporal autocorrelation function, which quantifies how similar the signal remains to itself after a delay time τ : $[G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F \rangle^2}]$ (Fig. 16). [153, 225, 288, 289] The amplitude of the autocorrelation function at short lag times

is inversely proportional to the average number of fluorescent molecules in the observation volume, $G(0) \propto 1/\langle N \rangle$, and therefore reports on local concentration. [153, 224, 225, 285, 290]

The decay of the autocorrelation function reflects the characteristic timescales of the processes responsible for the fluorescence fluctuations. For freely diffusing molecules in a three-dimensional Gaussian observation volume, the diffusion time τ_D is related to the diffusion coefficient D approximately by $\tau_D \sim \omega_0^2/4D$, where ω_0 is the lateral beam waist. [153, 225, 234, 289, 291] By fitting the autocorrelation curve with appropriate models, FCS can quantify diffusion coefficients, hydrodynamic size, local concentration, and diffusion modes, including three-dimensional diffusion, two-dimensional membrane diffusion, and anomalous diffusion. [153, 225, 288, 289, 292]

Importantly, the autocorrelation curve is not solely determined by diffusion. It is a composite of contributions from translational diffusion, photophysical processes such as triplet-state formation and blinking, and chemical or conformational reactions. [234, 262, 263, 281, 288, 289, 293, 294, 295, 296, 297, 298, 299, 300, 301] Triplet-state transitions and other reversible bright–dark switching processes typically contribute fast components in the submicrosecond to microsecond range. [263, 300] Static or dynamic quenching, photobleaching, and saturation at high excitation powers can distort amplitudes and apparent diffusion times if not explicitly included in the fitting model. [281, 297, 299]

FCS can also report on binding, unbinding, and reaction kinetics when these processes alter diffusion, brightness, FRET efficiency, or fluorescence on/off states. [268, 288, 291, 293, 302] In reaction–diffusion systems, analytical autocorrelation functions contain additional kinetic components whose amplitudes and decay times depend on association and dissociation rate constants, species brightness, and the relative fractions of free and bound states. [293, 294, 296, 298, 300] Thus, when an appropriate mechanistic model is available, FCS can extract equilibrium constants, bound fractions, lifetimes, and kinetic rate constants from equilibrium fluctuations without externally perturbing the system. [264, 268, 284, 293]

This sensitivity makes FCS highly versatile for studying molecular dynamics and interactions in vitro, in living cells, in membranes, and in more complex biological systems. [235, 303] It has been applied to diffusion and transport, molecular aggregation, chemical reaction kinetics, binding interactions, membrane organisation, photophysical relaxation, and fluorescence intermittency. [288, 289, 298, 304, 305] However, because FCS is inherently a statistical method, reliable quantitative interpretation requires fitting experimentally measured autocorrelation curves with models that account for all relevant fluctuation sources. [235, 289]

A key strength of FCS is that it operates under equilibrium or steady-state conditions and can access molecular dynamics over timescales from nanoseconds to seconds with single-molecule sensitivity. [153, 288] At the same time, careful distinction must be made between concentration fluctuations, diffusion, binding reactions, and photophysical effects, since each can contribute to the measured correlation signal. [235, 281, 285] Accurate FCS analysis, therefore, depends on selecting appropriate physical models, controlling experimental conditions, and recognising that the measured autocorrelation function represents the combined influence of molecular number fluctuations, fluorescence-state dynamics, and re-

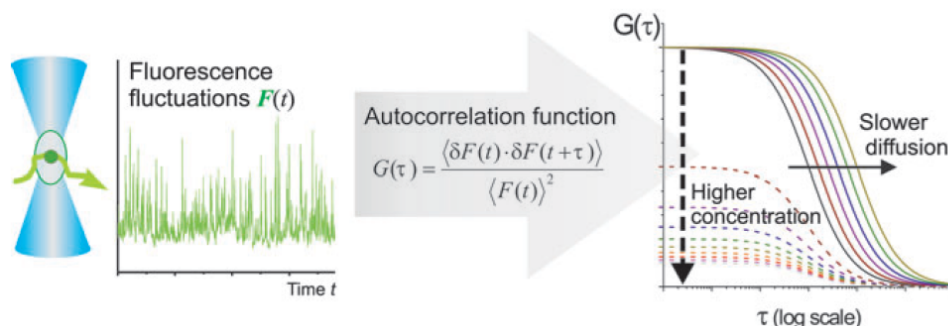


Fig. 16: Principle of fluorescence correlation spectroscopy (FCS). Fluorescently labelled particles diffuse through the confocal detection volume, producing intensity fluctuations over time. Autocorrelation analysis of the fluorescence trace yields the correlation function $G(\tau)$. The characteristic decay of $G(\tau)$ (solid curves) reflects the particle mobility, while the inverse of the amplitude at zero lag, $G(0)$, is proportional to the particle concentration (dashed curves). [283]

action kinetics.

Photophysical Effects in FCS

Fluorescence correlation spectroscopy (FCS) is a powerful and widely used technique for probing molecular dynamics, yet its measurements are inherently influenced by photophysical processes that modulate fluorescence emission. [301] In FCS, the recorded fluorescence intensity fluctuations reflect not only molecular diffusion and binding events but also intrinsic photophysical dynamics such as triplet-state population, blinking, and quenching. Because these processes directly affect the number of photons emitted per molecule, they play a central role in determining the apparent molecular brightness and must be explicitly considered in quantitative analyses.

Photophysical contributions are typically incorporated into the autocorrelation function (ACF) as additional components beyond diffusion. Fast fluorescence intermittency arising from triplet-state transitions is commonly modelled using an exponential term at short lag times, often in the sub-microsecond to microsecond range. [299] More generally, the ACF represents a composite of contributions from diffusion, photophysics, and chemical or conformational kinetics, and accurate interpretation requires fitting with models that explicitly include these distinct processes.

A key concept is that molecular brightness in fluctuation-based methods reflects the time-averaged detected emission per molecule. [261, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282] Any process that reduces fluorescence quantum yield or places the fluorophore in a non-emissive state lowers this effective brightness, whereas processes that shorten dark-state lifetimes can increase it. Thus, photophysical dynamics directly bias brightness-based measurements if not properly accounted for.

One of the most important photophysical processes is triplet-state formation. Following excitation, fluorophores may undergo intersystem crossing into a long-lived triplet state, which is non-emissive and can persist on microsecond timescales. This leads to fluorescence intermittency (“blinking”), where molecules switch between bright and dark states, reducing the average photon output. [261, 273, 274, 280] High triplet yields and long triplet lifetimes increase the steady-state population of dark states, thereby decreasing apparent brightness

and promoting photobleaching. [275, 276, 277]

Photobleaching is an irreversible loss of fluorescence caused by photochemical damage. It reduces the number of emitting molecules over time, leading to a decrease in signal intensity and distortion of the autocorrelation function. [275, 276, 277, 279] In FCS, photobleaching can affect both the amplitude and the shape of the ACF, particularly for slowly diffusing species or under high excitation intensities. Progressive depletion of fluorophores may produce apparent changes in concentration or diffusion behaviour if not properly corrected. [235] Moreover, triplet-mediated pathways can make photobleaching and brightness strongly dependent on excitation power, introducing nonlinear effects into the measurement. [275, 279, 280]

Quenching processes further complicate interpretation. Dynamic (collisional) or static quenching reduces fluorescence quantum yield without necessarily changing the number of fluorophores, thereby lowering brightness. [281] In FCS, this can mimic changes in concentration or diffusion if not explicitly modelled. At high local fluorophore concentrations, additional effects such as homo-FRET, triplet-triplet annihilation, and self-quenching can occur, reducing per-molecule brightness through intermolecular interactions. [282]

These photophysical processes collectively reshape the fluorescence signal detected in FCS. Triplet formation, blinking, photobleaching, and self-quenching all alter the duration and efficiency of fluorophore emission, thereby strongly influencing both molecular brightness and the form of the autocorrelation function. [261, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282] If not accounted for, they can lead to systematic errors in inferred parameters such as concentration, diffusion coefficients, and binding equilibria.

To address these challenges, both experimental and analytical strategies are employed. Experimentally, photophysical artefacts can be mitigated by optimising excitation power, using oxygen scavengers or triplet-state quenchers, and limiting acquisition to time windows preceding significant photobleaching. Analytically, explicit photophysical terms are incorporated into correlation or photon-counting models, allowing separation of true molecular dynamics from dye-related processes. [299, 301]

In addition, methodological advances have expanded FCS’s capabilities in complex systems. Techniques such as fluorescence cross-correlation spectroscopy (FCCS) and stimulated emission depletion FCS (STED-FCS) improve spatial resolution, reduce background, and enable analysis of interactions and dynamics in heterogeneous environments. [153] Despite these advances, careful consideration of photophysical effects remains essential, as they fundamentally determine the relationship between fluorescence fluctuations and the underlying molecular processes being studied.

Equilibrium Binding Studies with FCS

Fluorescence correlation spectroscopy (FCS) provides direct access to equilibrium properties of interacting molecular systems by analysing the relaxation of spontaneous concentration fluctuations under thermodynamic equilibrium. [294] In such systems, local concentrations fluctuate around their mean values due to coupled diffusion and reaction processes, and the temporal evolution of these fluctuations is governed by diffusion coefficients, reaction rate constants, and equi-

brium populations. All of these parameters are encoded in the fluorescence autocorrelation function ($G(\tau)$), which serves as the central observable in FCS.

For binding reactions, FCS extracts equilibrium constants by fitting the autocorrelation function with appropriate reaction–diffusion models. In limiting cases—where binding is either much faster or much slower than diffusion through the observation volume—analytical expressions for ($G(\tau)$) are well established. [294] More generally, the shape and decay of the autocorrelation curve reflect both molecular mobility and interconversion between bound and unbound states, enabling quantitative determination of equilibrium and kinetic parameters when suitable models are applied. [296]

In practice, equilibrium binding studies with FCS rely on monitoring changes in fluorescence fluctuations as a function of ligand or binding partner concentration. When a fluorescent ligand binds to a larger target such as DNA, protein, or a supramolecular complex, the resulting bound species typically diffuses more slowly. This shifts the autocorrelation decay toward longer lag times. By analysing this shift as a function of concentration and fitting it with a binding model, equilibrium association constants can be obtained. [232, 306, 307]

However, diffusion-based discrimination has inherent limitations. Binding events can only be resolved if the bound and unbound species differ sufficiently in their diffusion coefficients. [153] In many biologically relevant systems — particularly when small ligands bind to large macromolecules or when multiple ligands bind to the same target — the diffusion differences may be too small to distinguish clearly. In such cases, diffusion alone is insufficient to unambiguously characterise binding interactions. [235]

To overcome this limitation, molecular brightness analysis (MBA) and related fluctuation-based approaches are often combined with FCS. Brightness provides an independent observable that reflects the photon emission rate per diffusing particle and can therefore report on binding stoichiometry. [232, 264] For example, when multiple fluorescent ligands bind to a single DNA molecule, the brightness per complex increases (or changes in a characteristic way if quenching occurs), allowing inference of the number of bound molecules. By combining diffusion and brightness information within appropriate binding models, both equilibrium constants and binding stoichiometry can be determined. [232, 306, 308]

More advanced theoretical treatments show that the autocorrelation function can encode equilibrium constants even when binding alters fluorescence brightness. Reaction–diffusion models incorporate both mobility differences and brightness contrasts, enabling the extraction of thermodynamic parameters across a wide range of kinetic regimes. [294] In addition, higher-order correlation approaches and fluctuation-distribution analyses — such as photon counting histogram (PCH), fluorescence intensity distribution analysis (FIDA) — allow simultaneous determination of diffusion times, brightness, and concentrations of multiple species within a single experiment. [264]

Complementary FCS-based techniques further enhance the study of equilibrium binding. Dual-colour fluorescence cross-correlation spectroscopy (FCCS) labels interacting molecules with spectrally distinct fluorophores and directly measures their co-diffusion by cross-correlating the two detection channels. This approach provides a robust readout of complex formation that does not rely solely

on changes in diffusion time and is less susceptible to interference from nonspecific interactions. [153] Similarly, fluorescence lifetime correlation spectroscopy (FLCS) can separate species based on differences in fluorescence lifetime, improving discrimination in heterogeneous systems.

Despite these capabilities, extracting equilibrium constants from FCS requires careful modelling. Conventional second-order autocorrelation analysis may not uniquely determine all relevant parameters—such as equilibrium constants, rate constants, and brightness ratios — without additional constraints or complementary measurements. [309] Furthermore, when binding and unbinding occur on timescales comparable to diffusion, the resulting autocorrelation features become coupled, necessitating more sophisticated reaction–diffusion models to disentangle the contributions.

Overall, FCS provides a powerful framework for equilibrium binding studies by linking concentration-dependent changes in fluorescence fluctuations to underlying thermodynamic parameters. By combining diffusion analysis, molecular brightness, and advanced fluctuation methods within appropriate theoretical models, it is possible to quantitatively determine binding affinities, stoichiometry, and interaction mechanisms — even in complex, multi-site systems under near-native conditions.

Instrumental and Methodological Considerations

Modern FCS achieves single-molecule sensitivity by combining a confocal optical design with highly sensitive photon-counting detectors. Sub-femtoliter observation volumes are generated using high–numerical–aperture objectives and spatial filtering, while low-noise avalanche photodiodes enable reliable detection of fluorescence from only a few molecules within the detection volume at any given time. [262] These capabilities underpin the quantitative power of FCS but also place stringent demands on instrumental stability and calibration.

A central assumption in FCS measurements is that the system remains stationary during data acquisition, meaning that the statistical properties of the fluorescence signal do not change over time. [289] At the same time, quantitative interpretation relies on an accurate description of the observation volume, which is typically approximated as a three-dimensional Gaussian defined by the point spread function of the confocal microscope. In practice, however, this volume depends sensitively on optical alignment, refractive index matching, and aberrations in the excitation and detection paths. Deviations from the ideal volume shape can distort autocorrelation amplitudes and decay times, leading to systematic errors in inferred concentrations, diffusion coefficients, and molecular brightness.

Noise and statistical accuracy are intrinsic considerations in FCS. The signal-to-noise ratio is governed not only by instrumental factors but also by fundamental physical limits, including photon shot noise and the stochastic nature of molecular fluctuations themselves. [289] These contributions set the precision with which parameters can be extracted and require appropriate weighting schemes during fitting. Additional sources of noise, such as detector dark counts or electronic fluctuations, further influence measurement quality and must be minimised or corrected.

An important methodological point is that molecular brightness is not solely an intrinsic property of the fluorophore but depends strongly on the optical setup

and detection conditions. Factors such as excitation intensity, collection efficiency, detector quantum efficiency, and background fluorescence all contribute multiplicatively to the detected count rate per molecule. [154, 158, 298, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321] As a result, brightness values are only directly comparable when measured under identical experimental conditions, and quantitative interpretation requires careful calibration.

Excitation power must be carefully controlled because it influences both signal strength and photophysical behaviour. At low excitation intensities, fluorescence scales linearly with power, enabling straightforward interpretation of brightness and concentration. At higher powers, however, saturation effects, triplet-state accumulation, and photobleaching lead to nonlinear responses in which the count rate per molecule no longer increases proportionally with excitation. Background contributions such as scattering and Raman emission may continue to increase, reducing the effective signal-to-background ratio and distorting the autocorrelation function. For this reason, quantitative FCS measurements are typically performed in a regime where excitation remains sufficiently low to avoid these nonlinearities.

Detector characteristics also play a critical role. The overall detection efficiency determines the number of photons collected per molecule and thus directly affects brightness and measurement precision. Detector-specific effects, including dark counts, afterpulsing, gain calibration, and readout noise, can introduce systematic biases if not properly characterised. For example, elevated dark noise can artificially reduce correlation amplitudes, while incorrect gain calibration leads directly to errors in brightness and particle number estimation. Careful detector calibration and, where possible, optimisation such as cooling to reduce dark counts are therefore essential.

Background fluorescence and scattering represent another major source of systematic error. An uncorrelated background reduces the amplitude of the autocorrelation function, leading to an overestimation of the number of molecules and an underestimation of molecular brightness. This effect becomes particularly significant at low fluorophore concentrations or when studying weakly emitting species. Accurate measurements, therefore, require independent determination of background signals and appropriate corrections during data analysis.

Methodological extensions of FCS have been developed to address specific experimental limitations. Scanning FCS improves sampling of slow-diffusion processes by moving the observation volume across the sample, thereby increasing the number of independent events contributing to the signal. [153] Dual-focus and multifocal approaches enable simultaneous measurements at multiple spatial positions, while total internal reflection FCS restricts excitation to a thin evanescent field near an interface, reducing background and enabling studies of surface-associated dynamics. [153, 295] More advanced implementations, such as stimulated emission depletion FCS, reduce the effective observation volume and extend measurements to nanometre length scales.

For quantitative equilibrium and binding studies, calibration is indispensable. Accurate determination of molecular concentration requires knowledge of the effective observation volume, typically obtained using reference fluorophores with known diffusion coefficients. Similarly, molecular brightness must be related to a calibrated intensity scale, and detection efficiency must be accounted for to enable

comparison between experiments. Without such calibration, fluorescence measurements remain in arbitrary units and cannot be reliably interpreted as physical quantities, such as binding constants or stoichiometry.

Finally, experimental artefacts such as photobleaching, fluctuations in excitation intensity, and sporadic bright events from aggregates or impurities can distort the autocorrelation function and complicate data fitting. These effects can introduce apparent changes in diffusion or concentration if not properly accounted for. Careful experimental design, including optimisation of acquisition conditions and validation of fitting models, is therefore essential for obtaining reliable and reproducible results.

Overall, the accuracy of FCS and molecular brightness analysis depends critically on precise control of instrumental parameters, rigorous calibration, and careful treatment of noise and background contributions. Only under such conditions can fluorescence fluctuations be quantitatively related to the underlying molecular processes of interest.

Complementarity of FCS with Other Biophysical Methods

Fluorescence correlation spectroscopy is a versatile technique capable of quantifying molecular concentration, diffusion dynamics, and biomolecular interactions with high temporal resolution and single-molecule sensitivity. [153] By analysing fluorescence fluctuations in extremely small observation volumes, FCS provides access to diffusion coefficients, hydrodynamic radii, and interaction dynamics under equilibrium conditions, often at pico- to nanomolar concentrations. This exceptional sensitivity enables FCS to probe systems inaccessible to many ensemble techniques, particularly those involving low-abundance species, heterogeneous populations, or complex biological environments.

A defining strength of FCS is its ability to extract dynamic and equilibrium information simultaneously from spontaneous fluctuations. Diffusion coefficients and concentrations are obtained directly from autocorrelation analysis, while binding interactions can be inferred either from changes in diffusion behaviour or more directly through extensions such as FCCS, which detects co-diffusion of interacting species. [153, 292] Additional variants, including scanning FCS, fluorescence lifetime correlation spectroscopy, and super-resolution implementations such as STED-FCS, further extend the method by enabling spatial mapping of dynamics, lifetime-based discrimination of species, and access to nanoscale transport processes.

Despite these capabilities, FCS remains inherently model-dependent. Quantitative interpretation requires that the chosen autocorrelation model accurately captures all relevant processes contributing to the signal, including diffusion, photophysics, and chemical kinetics. [296] In complex systems where multiple species coexist or where different processes occur on similar timescales, separating contributions to the correlation signal can become challenging. In such cases, FCS alone may not provide a complete or unambiguous description of the system.

For this reason, FCS is most powerful when used in conjunction with complementary biophysical methods. Bulk techniques such as fluorescence titration, absorption spectroscopy, fluorescence anisotropy, and isothermal titration calorimetry (ITC) probe the same binding equilibria but operate in different concentration regimes and provide distinct types of information. Bulk methods typically yield

equilibrium constants, stoichiometry, and thermodynamic parameters with high precision, but they average over large ensembles and may obscure heterogeneity or rare states. In contrast, FCS directly probes fluctuations of small molecular ensembles, enabling detection of multiple coexisting states, transient intermediates, and rare species that are often invisible to ensemble measurements.

These approaches are therefore complementary rather than interchangeable. FCS excels at low concentrations, in heterogeneous or crowded environments, and in systems where kinetic or dynamic information is essential. For example, it can quantify binding interactions in complex biological media, detect rare aggregates present at extremely low abundance, and resolve heterogeneity within nominally identical complexes. At the same time, bulk techniques remain indispensable for obtaining comprehensive thermodynamic descriptions, performing high-throughput measurements, and validating equilibrium constants under well-controlled conditions.

Integration of FCS with other methods often improves both accuracy and interpretability. For instance, bulk absorption or fluorescence measurements can be used to determine labelling efficiency or concentration, which can then be incorporated into FCS-based binding models. Conversely, FCS can provide kinetic or heterogeneity information that complements equilibrium parameters obtained from ensemble techniques. In practice, combining fluctuation-based and bulk approaches allows cross-validation of binding constants and stoichiometry while reducing the impact of method-specific artefacts.

It is also important to recognise that both FCS and bulk methods are susceptible to distinct sources of error. In FCS, photobleaching, blinking, and uncertainties in the calibration of the observation volume can bias results if not properly corrected. Bulk techniques may suffer from artefacts related to sample preparation, aggregation, or indirect readouts of binding. Discrepancies between methods are therefore not uncommon and often require careful analysis to reconcile.

Overall, the complementarity of FCS with other biophysical techniques lies in the balance between sensitivity, resolution, and scope. FCS provides unparalleled access to molecular dynamics, heterogeneity, and low-abundance species at the single-molecule level, while ensemble methods offer robust thermodynamic characterisation and broader experimental throughput. Together, these approaches provide a more complete and reliable understanding of biomolecular interactions than either could achieve alone.

1.5 Brightness Analysis Method

Concept of Molecular Brightness

Single-molecule fluorescence techniques have transformed the study of biomolecular systems by enabling direct observation of individual particles rather than ensemble-averaged behaviour. Within this framework, it is essential to clearly distinguish among three related but fundamentally different quantities: fluorescence intensity, concentration, and molecular brightness. Intensity represents a bulk signal corresponding to the total number of detected photons, while concentration reflects the number of molecules per unit volume. In contrast, molecular brightness is a per-molecule property defined as the photon emission rate of a single fluorescent species under specified experimental conditions. [158, 160, 224, 229, 264, 266, 322, 323, 324, 325] As such, molecular brightness provides a direct link between observed fluorescence and the underlying photophysical behaviour of individual molecules.

In fluorescence fluctuation methods, including fluorescence correlation spectroscopy, the measured signal arises from many individual emitters contributing photons over time. A central concept is that the total fluorescence intensity can be expressed as the product of the number of emitting particles and their average molecular brightness. While the mean intensity depends on both factors simultaneously, fluctuation statistics—such as the signal variance or photon-count distribution—depend on particle number and brightness in different ways. [160, 322, 323, 324, 326, 327, 328, 329] This distinction is crucial because it allows molecular brightness and particle number to be extracted independently from the same fluorescence data, providing access to single-molecule properties even in ensemble measurements.

Molecular brightness is inherently dependent on experimental conditions, including excitation intensity, optical collection efficiency, and detector performance. At the same time, it reflects intrinsic photophysical properties of the fluorophore, such as absorption cross-section, quantum yield, and the population of dark or quenched states. Consequently, molecular brightness is not a universal constant but a state-specific parameter that can vary with local environment, molecular conformation, or binding interactions. Changes in molecular brightness, therefore, provide a sensitive readout of alterations in photophysics induced by processes such as ligand binding, conformational transitions, or aggregation.

This state dependence underlies the power of molecular brightness analysis (MBA). Because each molecular species — such as a free fluorophore, a bound complex, or an oligomeric assembly — can exhibit a distinct per-molecule brightness, MBA enables discrimination between species that may otherwise be indistinguishable by diffusion or spatial localisation alone. [161, 297, 325, 330, 331, 332, 333, 334, 335, 336, 337] In ideal cases, oligomerisation leads to approximately integer scaling of brightness (e.g., dimers being roughly twice as bright as monomers), although deviations from this behaviour commonly arise due to quenching, energy transfer, or other photophysical interactions between closely spaced fluorophores.

Importantly, molecular brightness must be interpreted relative to well-defined experimental conditions. Comparisons between different molecular states are most meaningful when measurements are performed under identical excitation and detection settings, ensuring that observed differences reflect genuine changes in mo-

lecular properties rather than instrumental variations. Within these constraints, molecular brightness serves as a powerful quantitative parameter that links fluorescence observables to molecular stoichiometry, interaction states, and photophysical behaviour, making it a central concept in modern fluorescence fluctuation spectroscopy.

Extraction of Brightness in Fluorescence Correlation Spectroscopy

Molecular brightness in fluorescence fluctuation spectroscopy is not measured directly but inferred from the statistical properties of the detected photon stream. In all implementations, brightness is defined as the detected photon rate per molecule — typically expressed as photons per molecule per second or per sampling interval — and is obtained by combining information about total fluorescence intensity with estimates of the number of emitting particles. [160, 224, 269, 270, 322, 323, 328, 338, 339] Conceptually, this reflects the requirement that any valid estimate of brightness must be consistent with both the mean signal and the magnitude of its fluctuations.

Two complementary routes are commonly used to extract molecular brightness. In autocorrelation-based FCS, the average number of molecules in the observation volume is obtained from the amplitude of the autocorrelation function, while the mean fluorescence intensity is determined directly from the photon time trace. Molecular brightness then follows from the ratio of these quantities. [328] In contrast, photon-counting approaches such as PCH analysis and related cumulant or moment-based methods determine brightness by fitting the full distribution of photon counts recorded over defined time bins. [269, 270] Although these approaches differ in implementation, they rely on the same underlying fluctuations and therefore provide mutually consistent estimates of brightness when appropriate models are applied.

FCS and PCH thus extract complementary aspects of the same data set. FCS primarily analyses the temporal structure of fluctuations, yielding diffusion coefficients, molecular concentrations, and kinetic parameters such as triplet-state lifetimes or reaction rates. PCH and related methods instead analyse the amplitude distribution of fluctuations, which is directly sensitive to the photon output per molecule and therefore to molecular brightness. [269, 270] When applied together, these methods enable simultaneous determination of diffusion, concentration, and brightness, providing a more complete description of the system than either approach alone.

In practice, extracting molecular brightness requires careful modelling of the fluorescence signal. Theoretical descriptions typically assume that fluorescence fluctuations arise from stochastic variations in the number and position of molecules within a well-defined observation volume, often approximated by a three-dimensional Gaussian profile. [328] Under these conditions, the detected signal is treated as a stationary and ergodic process, meaning that its statistical properties do not change over time and that time averages are representative of ensemble averages. Each molecular species is assumed to have a well-defined and constant brightness, and the detected photon counts are assumed to scale linearly with fluorescence intensity, with shot noise following Poisson statistics.

These assumptions are essential for translating measured fluctuations into physically meaningful brightness values, but they are not always strictly satisfied in

real experiments. Deviations can arise from photobleaching, slow intensity drift, detector artefacts, non-Gaussian observation volumes, or the presence of multiple unresolved species with different brightness values. [267, 270, 323, 340] In addition, the choice of binning time in photon-counting analyses introduces further constraints: if dynamic processes occur on timescales comparable to the bin width, the assumption of constant brightness within each bin may break down, leading to averaged or biased estimates.

A further challenge lies in correctly specifying the number of contributing species. Overly simplified models may merge distinct populations into a single effective brightness, whereas over-parameterised models can produce unstable or non-unique solutions. Consequently, reliable extraction of molecular brightness requires not only high-quality experimental data but also careful validation of the underlying assumptions and, where necessary, the incorporation of corrections for background signals, detector non-idealities, and deviations from idealised observation-volume geometry.

Despite these challenges, when properly implemented, fluorescence fluctuation methods provide a robust and internally consistent framework for determining molecular brightness. By linking photon statistics and autocorrelation analysis, these approaches enable quantitative extraction of per-molecule emission rates and thereby open a direct window onto molecular stoichiometry, interaction states, and photophysical behaviour in complex systems.

Brightness as a Reporter of Molecular States

Molecular brightness serves as a highly sensitive reporter of molecular states because it directly reflects the photon emission rate of individual fluorophores and therefore responds to both changes in stoichiometry and alterations in the local photophysical environment. In molecular brightness analysis (MBA), fluorescence intensity fluctuations are modelled as arising from a mixture of species, each characterised by a distinct brightness and population. By fitting these fluctuations, MBA resolves multiple brightness components that correspond to different molecular states, such as free versus DNA-bound species. [229, 323, 324, 325, 337, 341, 342, 343, 344] Because DNA binding often modifies quantum yield — either enhancing or quenching fluorescence — the resulting shift in emission efficiency produces clearly distinguishable brightness populations that can be quantitatively analysed.

These brightness changes originate from modifications in the fluorophore’s micro-environment upon binding or hybridisation. Interactions with DNA can alter polarity, rigidity, and non-radiative decay pathways, including proton- or electron-transfer processes, thereby affecting both quantum yield and, in some cases, absorption properties. Such effects are widely observed with fluorescent base analogues, intercalators, and groove-binding dyes, in which duplex formation or binding induces measurable changes in molecular brightness. [337, 343, 344] MBA translates these photophysical differences into distinct brightness classes, enabling direct quantification of bound and unbound fractions without the need for physical separation of species. [229, 324, 341, 342] In this framework, any environment- or binding-induced change in fluorescence output appears as a separable component in the brightness distribution.

In addition to reporting on binding, molecular brightness encodes information

about stoichiometry. For fluorophore-labelled biomolecules, brightness scales approximately with the number of emitting labels when photophysical interactions are minimal. Thus, dimers are typically about twice as bright as monomers, and higher-order oligomers scale accordingly. [331, 332] This relationship allows assignment of observed brightness levels to specific oligomeric states when calibrated against known standards, such as monomeric probes or engineered oligomers with defined fluorophore numbers. [297, 331, 332, 333] In more complex systems, changes in stoichiometry upon binding — such as protein oligomerisation on DNA — manifest as new brightness populations that may also be associated with altered diffusion properties, enabling simultaneous resolution of size and composition.

Importantly, brightness is influenced not only by the number of fluorophores but also by their photophysical behaviour. Binding events that do not change stoichiometry can still produce distinct brightness states if they alter quantum yield, dark-state populations, or other emission properties. For example, DNA intercalation often constrains fluorophore motion and reduces non-radiative decay, thereby enhancing fluorescence, whereas other binding modes may introduce quenching pathways. In such cases, the bound complex appears as a distinct brightness species even though the number of fluorophores remains unchanged. [330, 331, 337] This dual sensitivity to both stoichiometry and environment makes brightness a particularly versatile observable.

A major strength of brightness-based approaches lies in their analysis of full intensity distributions rather than mean signals. In a homogeneous system, the photon-count histogram is narrow and fully described by a single brightness and a single particle number. [160, 328] In heterogeneous systems, however, the overall distribution becomes broader or asymmetric, reflecting the superposition of contributions from multiple species with different brightness values. [160, 264, 324, 328] By fitting these distributions with multi-component models — such as photon counting histogram, fluorescence intensity distribution analysis, or related methods — it is possible to extract both the brightness and concentration of each species, thereby revealing heterogeneity in binding, oligomerisation, or local environment. [160, 264, 324, 345]

Extensions of brightness analysis to spatially resolved measurements further highlight its utility as a reporter of molecular states. Imaging-based approaches, including Number and Brightness or spatial intensity distribution analysis, generate maps or distributions of brightness across many pixels or regions of interest. Variations in these distributions indicate spatial heterogeneity in oligomerisation or local environment, while clustering or multimodal behaviour reveals the presence of distinct molecular populations. [323, 325, 346, 347] Such analyses can uncover rare or transient species that do not dominate average intensity but contribute characteristic brightness signatures.

Taken together, these features establish molecular brightness as a powerful and multifaceted reporter of molecular states. By linking photon emission to both fluorophore number and photophysical environment, brightness-based methods enable quantitative identification of binding events, determination of oligomeric states, and detection of heterogeneity within complex systems, all within a unified fluctuation-based framework.

Photophysical and Experimental Influences on Brightness

Brightness-based analyses in fluorescence fluctuation spectroscopy assume that each molecule emits photons at a constant rate; however, real fluorophores deviate substantially from this idealised behaviour. Processes such as blinking, photobleaching, energy transfer, and aggregation dynamically modulate photon emission, thereby altering both the average number of photons emitted per molecule and the temporal structure of the detected signal. [261, 273, 279, 281, 338, 348, 349, 350, 351, 352, 353] As a result, the apparent molecular brightness — and any quantities derived from it, such as oligomerisation state or concentration — can be significantly biased if these effects are not explicitly accounted for.

A major contributor to brightness variability is the presence of long-lived dark states, most notably triplet states and other non-emissive configurations. Many dyes and fluorescent proteins undergo intersystem crossing or reversible transitions into such states, leading to intermittent fluorescence (“blinking”). [261, 281, 351, 352] In fluctuation-based measurements, time spent in dark states reduces the time-averaged brightness per molecule and introduces additional short-timescale components in the autocorrelation function. If neglected, these effects can lead to systematic underestimation of brightness or misinterpretation as changes in particle number or diffusion behaviour. [281, 352] Moreover, triplet-state dynamics are strongly dependent on excitation power and the local chemical environment, which in turn influence both the effective photon budget and the apparent photostability of the fluorophore. [273, 279]

Irreversible photobleaching introduces further complexity by progressively removing fluorescent molecules from the observation volume. In confined systems such as living cells, this “photodepletion” leads to non-stationary signals that violate a central assumption of fluctuation analysis and bias brightness estimates even when overall intensity loss appears modest. [338] In multimeric systems, partial photobleaching can convert a uniformly bright population into a heterogeneous mixture of fully labelled, partially labelled, and non-fluorescent species. For example, a dimer initially exhibiting twice the brightness of a monomer may, upon bleaching of one fluorophore, contribute to a lower-brightness population, thereby skewing the inferred oligomer distribution toward smaller assemblies. [338] Photo-conversion processes can produce similar effects by generating alternative emissive states with distinct brightness values, further complicating interpretation if not incorporated into the analysis model. [338, 348]

Additional photophysical processes, including static and dynamic quenching, homo-FRET, and aggregation, further decouple the relationship between fluorophore number and detected photon output. Quenching mechanisms reduce the quantum yield without changing the number of molecules, leading to decreased brightness and broader intensity distributions. [281] In multichromophoric systems, interactions such as energy transfer or singlet–singlet annihilation can induce collective blinking behaviour, altering photon statistics and potentially leading to incorrect estimates of molecule number in fluctuation-based analyses. [350] Aggregation-caused quenching (ACQ) is particularly relevant in densely labelled systems or nanoparticles, where close packing promotes non-radiative decay pathways and results in particles that are significantly dimmer than expected based on fluorophore count. [353] Collectively, these effects introduce substantial

deviations from ideal brightness scaling and can mimic changes in concentration, diffusion, or oligomerisation.

In parallel with intrinsic photophysics, experimental conditions strongly influence measured brightness. Accurate interpretation of fluorescence fluctuations relies on several key assumptions, including linear detection, well-characterised noise, and a known observation volume profile. Detected photon counts are generally assumed to follow Poisson statistics and to scale linearly with fluorescence intensity, as described by Mandel’s formalism. [328] Deviations from this behaviour — arising from detector dead time, afterpulsing, or nonlinearity — can distort brightness estimates if not corrected. Background signals, including autofluorescence and detector noise, reduce the effective correlation amplitude and lead to overestimation of molecule number and underestimation of brightness unless properly subtracted. [270, 323, 340]

Equally important is the spatial definition of the observation volume, typically approximated as a three-dimensional Gaussian point spread function (PSF). [270, 328, 354] Deviations from this idealised profile — due to optical aberrations, misalignment, or inappropriate pinhole settings — can alter both the apparent number of molecules and their inferred brightness. Excitation power must also be carefully controlled: while increasing power initially enhances photon count rates, it simultaneously promotes triplet-state accumulation, saturation, and photobleaching, leading to non-linear and often misleading brightness behaviour.

Taken together, these considerations highlight that molecular brightness is not solely an intrinsic property of a fluorophore or molecular complex but rather an emergent quantity shaped by both photophysical processes and experimental conditions. Reliable quantitative interpretation, therefore, requires careful experimental design, rigorous calibration, and explicit incorporation of relevant photophysical and instrumental effects into the analysis framework.

Application to Equilibrium Binding Studies

Molecular brightness analysis provides a powerful framework for extracting equilibrium binding parameters directly from fluorescence fluctuation data by exploiting changes in per-molecule photon output rather than relying solely on diffusion behaviour. Even when differences in brightness between free and bound states are relatively small — on the order of a few percent — MBA can resolve these changes and quantify binding equilibria at extremely low concentrations, typically in the picomolar to nanomolar range. [229] In such cases, binding events are translated into distinct brightness populations, corresponding, for example, to free versus DNA-bound species, enabling determination of the bound fraction without the need for physical separation. [229, 324, 341, 342]

In practical applications, the MBA combines fluorescence intensity, autocorrelation amplitude, and photon-count statistics to fit experimental data with appropriate binding models. In ligand–protein or dye–DNA titrations, the observed signal is modelled as a mixture of species with distinct molecular brightness values, such as free ligand, singly bound complexes, or higher-order assemblies. By analysing how both the average intensity and the autocorrelation amplitude vary with concentration, it becomes possible to extract dissociation constants, binding stoichiometry, and, in some cases, the presence of multiple coexisting bound states. [330, 331] Importantly, MBA-based approaches can simultaneously account

for differences in diffusion and brightness, allowing these contributions to be disentangled even when they are partially overlapping. [229, 323, 324, 342]

A key advantage of brightness-based analysis is its sensitivity to photophysical changes induced by binding. Many fluorescent dyes exhibit substantial increases in quantum yield upon interaction with nucleic acids due to restricted conformational flexibility or reduced solvent quenching. As a result, the transition from free to bound state is accompanied by a measurable increase in molecular brightness, which can be directly related to the fraction of bound molecules. [229, 341] This principle extends to a wide range of systems, including intercalating dyes, groove binders, and fluorescent base analogues, where environmental changes such as polarity, rigidity, and hydrogen-bonding networks modulate fluorescence efficiency. In such systems, MBA effectively converts photophysical sensitivity into a quantitative readout of binding equilibria.

Beyond simple two-state models, MBA can be applied to more complex binding scenarios. For example, cooperative binding and neighbour-exclusion effects — commonly described by models such as McGhee–von Hippel or Hill-type formalisms — can be incorporated by assigning distinct brightness values to different binding states and fitting the resulting brightness versus concentration curves. [188, 341] Experimental studies on anthracycline–DNA interactions have demonstrated that MBA can resolve multiple equilibrium states with different fluorescence efficiencies, revealing inflexion points in brightness that correspond to distinct binding modes. [188] In principle, any thermodynamic model that predicts the distribution of bound states can be integrated into a brightness-based framework, provided that those states exhibit distinguishable photon emission characteristics.

The strength of MBA becomes particularly evident in systems where diffusion-based FCS analysis is insufficient. When binding partners have similar hydrodynamic radii, the resulting diffusion times may overlap, preventing reliable separation of free and bound species using conventional correlation analysis. In contrast, brightness-based methods distinguish species based on photon output rather than mobility, enabling resolution of mixtures that are otherwise indistinguishable. [355] Because molecular brightness scales with the number of fluorophores per complex and is sensitive to environmental effects, it provides a direct link to binding stoichiometry and molecular state. This allows discrimination between, for example, single-site and multi-site binding, as well as quantification of heterogeneous populations within the same sample. [264, 330]

Furthermore, photon-counting and higher-order fluctuation analyses enhance sensitivity to brightness differences beyond what is achievable with standard second-order autocorrelation alone. These approaches improve the resolution of multiple reactive species and enable the detection of minor populations that contribute only weakly to the overall signal. [264, 356] As a result, MBA offers a complementary and often superior route to determining equilibrium constants in complex systems, particularly when combined with appropriate physical models and careful experimental calibration.

Overall, molecular brightness analysis extracts binding information from how the per-molecule photon emission changes with system composition. By leveraging differences in fluorescence efficiency between molecular states, MBA enables

quantitative determination of binding affinities, stoichiometries, and interaction mechanisms, even when traditional diffusion-based or ensemble methods provide limited or ambiguous information.

Instrumental Considerations and Calibration

Quantitative interpretation of molecular brightness requires rigorous calibration and careful control of the optical and detection system, as the measured photon count per molecule is not an intrinsic constant but an experimentally defined quantity. In practice, brightness is often referenced to well-characterised standards, such as monomeric and oligomeric fluorescent proteins, enabling assignment of measured brightness levels to specific oligomeric states. For example, calibration using constructs such as tandem Enhanced Green Fluorescent Protein oligomers allows direct comparison of monomers, dimers, and higher-order assemblies, with brightness scaling approximately linearly under controlled conditions. [297, 331, 332, 333] Such calibration strategies provide a bridge between measured fluorescence fluctuations and absolute copy numbers per complex, although their accuracy remains limited by signal-to-noise ratio and experimental variability. [162, 357]

The validity of brightness measurements relies on a set of underlying assumptions regarding the measurement system and the sample. These include fluorophore homogeneity, equilibrium conditions, and a well-defined observation volume, typically approximated by a three-dimensional Gaussian point spread function. [270, 328, 354] Deviations from this idealised profile—arising from optical aberrations, misalignment, or refractive index mismatches—can distort both the apparent number of molecules and their inferred brightness. Similarly, detection must remain linear, with photon counts proportional to fluorescence intensity and governed by Poisson statistics, while detector-specific effects such as dead time, afterpulsing, and background noise must be either negligible or explicitly corrected. [270, 323, 328, 340]

Excitation conditions play a particularly critical role. Under low-power illumination, molecular brightness typically increases linearly with laser power, reflecting a proportional increase in fluorophore excitation. However, at higher excitation intensities, saturation effects, triplet-state accumulation, and photobleaching lead to non-linear behaviour, causing apparent brightness to plateau or even decrease. [265, 358] Accurate measurements, therefore, require operation within a well-defined linear regime, which is commonly established through power series experiments. In addition, the spatial profile of the excitation beam influences brightness, as molecules experience a non-uniform intensity distribution within the observation volume, effectively weighting their contribution to the detected signal. [323]

A precise definition of the confocal observation volume is equally essential, as brightness is defined per molecule within this volume and used in conjunction with it to determine concentration. The effective volume depends sensitively on optical alignment, numerical aperture, pinhole size, and aberrations, and inaccuracies in its characterisation can lead to systematic errors in both molecular number and brightness. [317, 327, 358] For this reason, calibration with reference dyes of known diffusion coefficients is often employed to determine the effective volume prior to quantitative measurements.

Detector performance further constrains brightness accuracy. The overall detection efficiency — determined by optical transmission and detector quantum efficiency — directly scales the measured photon count rate. High-efficiency detectors improve both signal strength and statistical precision, whereas noise sources such as dark counts, readout noise, and gain fluctuations introduce uncertainty and bias. [327] In single-photon avalanche diode (SPAD) systems, dark noise and afterpulsing can significantly affect fluctuation statistics, although these effects can be mitigated through cooling and appropriate correction algorithms. [319, 359]

Background contributions from autofluorescence, scattering, immobile structures, and detector noise represent an additional source of systematic error. These signals do not scale with molecular brightness in the same way as true fluorescence fluctuations, thereby reducing the apparent correlation amplitude and leading to overestimation of molecule number and underestimation of brightness if not properly accounted for. [321, 323] Accurate background measurement and subtraction are thus essential for reliable analysis.

Overall, the precision and accuracy of molecular brightness measurements are fundamentally limited by signal-to-noise ratio, which depends on photon flux, optical alignment, and detector characteristics. Reliable quantitative analysis requires operation in a non-saturating excitation regime, careful calibration of the observation volume, thorough characterisation of detector performance, and explicit correction for background signals. Failure to control these factors can lead to substantial errors in inferred brightness values and, consequently, in conclusions regarding molecular concentration, stoichiometry, and interactions.

Limitations and Complementarity of Molecular Brightness Analysis

Despite its considerable strengths, molecular brightness analysis is subject to important limitations that arise from both theoretical assumptions and practical constraints. A central assumption in fluorescence fluctuation methods is that each molecular species can be described by a single, well-defined brightness value. In reality, however, many systems exhibit multiple emissive states or broad brightness distributions due to heterogeneous environments, conformational dynamics, or photophysical variability. [154, 155, 156, 158, 160, 224, 360, 361] When such heterogeneity is present, the mapping between brightness and molecular state becomes non-unique, and standard analysis approaches may yield biased or ambiguous results.

The ability of the MBA to resolve distinct species depends critically on brightness contrast and photon statistics. Reliable separation typically requires sufficiently large differences in brightness—often on the order of a factor of two or greater under favourable conditions. [154, 160, 360] As the number of coexisting species increases, or as their brightness values become more similar, the accuracy of extracted concentrations and brightness values degrades. In such cases, even a minor population of brighter species can disproportionately influence the fluctuation signal, leading to distorted estimates of both molecular number and diffusion properties. [158] Furthermore, statistical analyses such as photon-counting histograms can exhibit non-linear behaviour when brightness or concentration distributions are broad, complicating model fitting and interpretation. [224]

Additional challenges arise from photophysical effects and experimental artefacts. Systems with strong blinking, intensity fluctuations, or excitation-dependent

behaviour can violate the assumptions underlying standard fluctuation analysis, producing apparent changes in brightness or diffusion that do not reflect true molecular properties. [155] Fluorescent probes that populate multiple emissive states—such as certain fluorescent proteins or photoconvertible dyes — require explicit multi-state models, as single-state descriptions can lead to incorrect assignments of oligomerisation or binding. [156, 361] Even advanced analytical approaches, including photon distribution analysis (PDA), cumulant-based methods, and global fitting strategies, are ultimately limited by photon statistics, parameter degeneracy, and computational complexity. [154, 155, 156, 224] As a consequence, the number of independently resolvable parameters is constrained, and over-fitting or non-unique solutions may occur in complex systems.

These limitations define the conditions under which MBA yields reliable results. The method is most robust when brightness states are well separated, photon counts are high, photophysical artefacts are minimised or modelled explicitly, and the number of assumed species matches the true system complexity. In scenarios involving rapid interconversion between states or strongly overlapping brightness distributions, the measured signal may represent an averaged effective state rather than distinct populations, reducing the interpretability of the results.

For this reason, the MBA is most powerful when used in combination with complementary biophysical techniques that probe orthogonal observables. Methods such as fluorescence anisotropy, absorption or fluorescence titration, and calorimetric approaches provide independent access to binding equilibria, stoichiometry, and thermodynamic parameters. For example, fluorescence anisotropy reports on rotational dynamics and molecular size, while isothermal titration calorimetry yields direct measurements of binding enthalpy and stoichiometry. MBA complements these ensemble techniques by providing single-molecule sensitivity and resolving heterogeneous populations that may be obscured in bulk measurements. In dye–DNA systems, brightness-based analyses have revealed multiple binding states and equilibrium regimes that are difficult to detect using conventional intensity or anisotropy measurements alone. [188]

The integration of MBA with other approaches enables cross-validation of binding constants and mechanistic models. Bulk spectroscopic methods offer high precision and throughput, whereas MBA provides detailed insight into molecular heterogeneity, stoichiometry, and rare species at low concentrations. Discrepancies between methods can often be traced to underlying heterogeneity or model assumptions, and MBA can help identify and resolve these complexities. In this way, molecular brightness analysis does not replace established techniques but rather extends their capabilities, contributing a uniquely sensitive and complementary perspective on biomolecular interactions and equilibria.

1.6 Continuous fluorescence photobleaching

Fundamentals of Photobleaching

Photobleaching is the irreversible loss of fluorescence from a fluorophore caused by photochemical damage following optical excitation. At the molecular level, this process originates from reactions occurring in electronically excited states, where the absorbed energy is diverted from radiative emission into pathways that generate chemically reactive intermediates. These intermediates — often formed via triplet-state involvement — ultimately lead to permanent structural modification of the fluorophore and to its loss of emissive capability. [277, 362, 363, 364, 365]

The photobleaching process begins with photon absorption, which promotes the fluorophore from the ground state to the first excited singlet state (S_1). From this state, the molecule may return to the ground state via fluorescence or undergo competing non-radiative pathways. A key competing process is intersystem crossing (ISC), in which the molecule transitions from S_1 to the triplet state (T_1) (Fig. 17). [277, 362, 363, 364] The triplet state is long-lived relative to singlet states, significantly increasing the probability of chemical interactions with the surrounding environment. As a result, it serves as a central intermediate in many photobleaching pathways.

Under typical experimental conditions, one dominant mechanism involves interaction between the triplet-state fluorophore and molecular oxygen. Energy transfer from T_1 to ground-state oxygen generates singlet oxygen and other reactive oxygen species (ROS), which can oxidise the fluorophore or initiate radical-mediated reactions that irreversibly disrupt its molecular structure. [277, 362, 364] These oxygen-dependent pathways are widely recognised as major contributors to photobleaching in biological and aqueous environments.

However, photobleaching is not exclusively oxygen-driven. Even under low-oxygen or deoxygenated conditions, fluorophores can degrade via alternative mechanisms. These include photoionisation from excited singlet or higher-energy states, leading to the formation of radical cations or anions that subsequently participate in destructive chemical reactions. [362, 365] In addition, sequential photon absorption — particularly under high-intensity or multiphoton excitation — can populate higher excited states, dramatically increasing the likelihood of bond cleavage and irreversible chemical transformation. [362, 363] In such regimes, photobleaching may exhibit non-linear dependence on excitation intensity, reflecting the involvement of multi-photon processes and higher-order electronic transitions.

The rate of photobleaching is therefore not governed by a single factor but instead emerges from the interplay between excitation conditions, fluorophore properties, and the local environment. Increasing excitation intensity generally accelerates photobleaching by increasing the population of excited and triplet states. In some cases, especially under femtosecond or multiphoton excitation, bleaching rates can scale non-linearly with power due to sequential absorption and ionisation processes. [349, 363] At the same time, the chemical environment — such as oxygen concentration, solvent composition, and matrix properties — strongly influences bleaching kinetics by modulating triplet lifetimes, diffusion of reactive species, and accessibility of quenching pathways.

Importantly, different fluorophores exhibit markedly different photobleaching

behaviours even under identical conditions. These differences arise from variations in molecular structure, electronic configuration, and susceptibility to oxidation or radical formation. [362, 366, 367] While many dyes show strong oxygen-dependent bleaching, others display significant degradation even in oxygen-poor environments, indicating the coexistence of multiple parallel pathways. [365] In certain systems, oxygen can even play a protective role by quenching triplet states before they engage in damaging reactions, highlighting the complex and context-dependent nature of photobleaching processes. [367]

Overall, photobleaching is best understood as a set of competing photophysical and photochemical processes initiated in excited states and mediated by both intrinsic molecular properties and external environmental factors. Because these processes vary widely across fluorophores and experimental conditions, empirical characterisation and optimisation are essential to minimise photobleaching and ensure reliable fluorescence measurements.

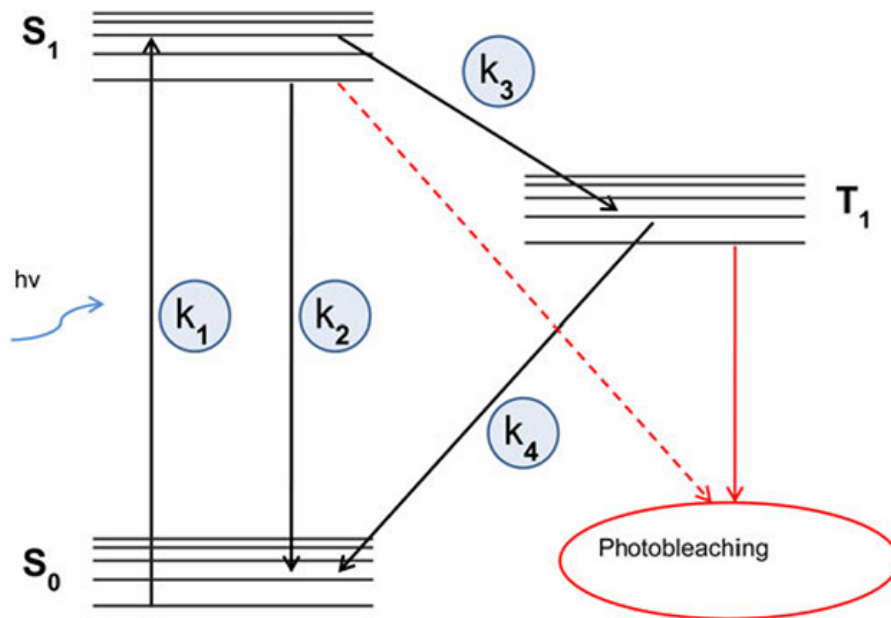


Fig. 17: A Jablonski diagram of a fluorophore in a three-level system illustrates its photophysical pathways. Upon excitation, the molecule transitions from the ground state (S_0) to the excited singlet state (S_1). From S_1 , the excess energy can be released through several competing processes, each defined by a specific rate constant (k). These include fluorescence emission, internal conversion, or intersystem crossing to the triplet state (T_1). Importantly, photobleaching primarily occurs from this long-lived triplet state (T_1), as the molecule in this state is more prone to undergo photochemical reactions that irreversibly destroy its fluorescence. [368]

Photobleaching Kinetics and Theoretical Models

Photobleaching is commonly described as a time-dependent decay of fluorescence intensity and is therefore amenable to kinetic modelling. In the simplest case, the loss of fluorescence follows first-order behaviour, yielding a mono-exponential decay of the form:

$$[I(t) = I_0 \exp(-kt)]$$

where (k) is the bleaching rate constant. This description is appropriate for homogeneous systems in which fluorophores experience similar excitation conditions and bleaching proceeds via unimolecular or pseudo-first-order processes, such as

reactions with a constant concentration of molecular oxygen. [277, 366, 369] Under such conditions, experimentally observed fluorescence decay often closely follows a single exponential, as demonstrated for simple dye systems at low concentration.

In more realistic environments, however, photobleaching kinetics frequently deviate from this idealised behaviour. Heterogeneity in the local environment — arising from variations in molecular binding, solvent accessibility, oxygen availability, or excitation intensity — leads to a distribution of bleaching rates across the fluorophore population. As a result, fluorescence decay is better described by multi-exponential models, in which the total signal is represented as a weighted sum of components with distinct rate constants. [277, 369, 370, 371] Such models can be derived from kinetic schemes that include multiple states, for example, emissive (“on”), non-emissive (“off” or blinking), and irreversibly bleached states, coupled through transition rates governed by photophysical and photochemical processes.

Even in systems where individual molecules exhibit uniform bleaching behaviour, ensemble measurements can display non-exponential decay due to spatial effects. In confocal or wide-field microscopy, fluorophores experience non-uniform illumination, typically described by a Gaussian intensity profile. Molecules located near the centre of the excitation volume are exposed to higher intensities and bleach more rapidly than those at the periphery, leading to an apparent distribution of rate constants and a deviation from single-exponential decay. [372] In such cases, the observed fluorescence decay can often be approximated by a sum of exponentials or, more generally, by stretched-exponential functions or continuous rate distributions. [373]

Additional complexity arises from higher-order photophysical interactions. In densely labelled systems or at high excitation intensities, bimolecular processes, such as triplet–triplet or triplet–ground-state interactions, contribute to bleaching kinetics, further deviating from simple first-order behaviour. [277, 369] These interactions can introduce non-linear dependencies on excitation intensity and concentration, complicating the interpretation of decay curves and requiring more sophisticated kinetic models.

Despite these complexities, several key parameters remain central to the quantitative description of photobleaching. These include the bleaching rate constant, which characterises the speed of fluorescence decay; the fluorescence half-life, defined as the time required for the intensity to decrease by half; and the photon budget per molecule, representing the total number of photons emitted prior to irreversible bleaching. These parameters provide practical metrics for comparing fluorophore performance and optimising experimental conditions.

In summary, while first-order exponential decay provides a useful baseline model for photobleaching in simple systems, real experimental data often require multi-exponential or non-exponential descriptions to account for environmental heterogeneity, complex photophysics, and spatially varying excitation. Accurate modelling of photobleaching kinetics, therefore, depends on selecting an appropriate level of theoretical complexity that reflects the underlying physical processes governing fluorophore degradation.

DNA substrate and immobilisation in agarose gel

λ -DNA serves as a well-established model substrate for fluorescence studies due to its defined length, known sequence composition, and reproducible physical properties. Its large size and uniform structure make it particularly suitable for investigating dye–DNA interactions and photophysical behaviour under controlled conditions.

In the present assay, λ -DNA is immobilised within a 5% agarose gel matrix. The agarose network restricts translational diffusion of the DNA molecules while maintaining a hydrated, quasi-physiological environment. This immobilisation is critical for enabling prolonged fluorescence observation and continuous photobleaching measurements without loss of molecules from the focal volume.

Compared with free DNA in solution, immobilisation within agarose suppresses diffusive exchange, ensuring that fluorescence decay arises predominantly from photobleaching rather than from molecular motion. The gel matrix may also influence local oxygen diffusion and microviscosity, thereby modulating photobleaching dynamics. For quantitative analysis, immobilisation is therefore essential to decouple photobleaching kinetics from transport effects.

Continuous Photobleaching as a Quantitative Method

Continuous photobleaching provides a quantitative framework in which the gradual loss of fluorescence under steady illumination is interpreted as a direct readout of the number of active, emissive species in a system. In fluorescence assays where only intact or properly bound complexes contribute to the signal, the time-dependent decrease in fluorescence intensity reflects the progressive depletion of these active species. By selecting an appropriate kinetic model, the shape and rate of fluorescence decay can therefore be mapped onto the number and state of fluorescently competent molecules. [342, 374, 375]

Because fluorescence intensity is proportional to the number of emitting fluorophores, monitoring its continuous decay enables tracking of the population of active complexes over time. Under controlled conditions, analysis of the decay curve — often in combination with statistical or kinetic modelling, and sometimes complemented by fluctuation-based methods such as fluorescence correlation spectroscopy — can yield quantitative information on stoichiometry, labelling density, and the fraction of functional complexes. [342, 374, 375] In this sense, continuous photobleaching transforms a loss process into a measurable proxy for molecular counting and population dynamics.

The validity of this approach, however, relies on several simplifying assumptions that assume a direct relationship between fluorescence intensity and molecular number. A common starting point is the assumption of first-order or pseudo-first-order bleaching kinetics, in which each fluorophore bleaches independently with a constant rate under fixed illumination conditions. [366, 370, 372, 373, 376, 377] Under this assumption, the surviving fraction of fluorescent molecules decays exponentially with time. Equally important is the assumption of a linear relationship between fluorescence intensity and the number of unbleached molecules, implying that each fluorophore contributes a constant molecular brightness throughout the measurement. [342, 372, 373, 377]

Additional constraints concern the uniformity of experimental conditions. Ideally, all molecules experience the same excitation intensity and chemical environ-

ment, ensuring that bleaching rates are homogeneous across the population. In practice, spatial variations in illumination — such as those arising from a Gaussian beam profile — can introduce a distribution of bleaching rates, leading to non-exponential decay that no longer corresponds to a single molecular lifetime. [372, 378] Similarly, reversible transitions into non-emissive (dark) states must either be negligible or explicitly incorporated into the kinetic model. If long-lived blinking states are present and not accounted for, temporary signal loss may be misinterpreted as irreversible photobleaching. [370, 379]

Within these assumptions, useful quantitative relationships emerge. For example, in systems described by mono- or multi-exponential decay, the time-integrated fluorescence emission — often referred to as the photon budget — can be related to the product of the initial intensity and the characteristic decay time, and may remain independent of excitation intensity under linear conditions. [372, 373, 380] This property enables comparison of fluorophore performance and estimation of the total number of emitted photons per molecule prior to bleaching.

When these simplifying assumptions hold, continuous photobleaching curves can be directly interpreted in terms of the number of photobleached molecules and the kinetics of their loss. However, deviations from ideal behaviour — such as non-linear excitation, heterogeneous environments, strong blinking, or spatially varying illumination — break this direct correspondence and require more sophisticated kinetic and spatial modelling. [342, 366, 370, 372, 373, 376, 377, 378, 379, 380] In such cases, continuous photobleaching remains informative but must be analysed with models that explicitly account for the underlying complexity of the system.

Linking Photobleaching with Molecular Brightness Analysis (MBA)

In fluorescence correlation spectroscopy, molecular brightness is defined as the detected photon count rate per molecule, making it inherently sensitive to processes that alter either the number of emitting molecules or their emission efficiency. Photobleaching affects both quantities simultaneously: it irreversibly removes fluorophores from the emissive population and can also modify the apparent brightness of the remaining species through changes in photophysics or population composition. [271, 311, 338, 342, 380, 381, 382, 383, 384, 385]

Within the FCS framework, photobleaching therefore violates the assumption of stationarity that underlies most brightness analyses. As bleaching progresses, the number of molecules in the observation volume decreases, altering the autocorrelation amplitude, while the distribution of molecular brightness can evolve over time. This effect is particularly pronounced for multimeric or multiply labelled species, where partial bleaching converts brighter complexes into dimmer ones, thereby reshaping the brightness distribution and biasing estimates of oligomerisation or stoichiometry if interpreted using static models. [311, 338]

At high excitation intensities, additional non-linear effects emerge. Triplet-state accumulation, saturation, and bleaching-induced photophysical changes can reduce the effective photon output per molecule, leading to an apparent decrease in molecular brightness that is not solely attributable to a reduction in the number of molecules. Under such conditions, standard FCS-derived brightness values may underestimate the true per-molecule emission rate unless explicit corrections are applied. [271, 383] For these reasons, accurate molecular brightness analysis in the presence of photobleaching requires either careful experimental design or

explicit modelling. Strategies such as operating in the low-power linear regime, ensuring exchange of unbleached molecules into the observation volume, or employing scanning-based approaches can minimise bleaching-induced bias. Alternatively, incorporating bleaching kinetics into the analysis allows separation of true molecular brightness from time-dependent population loss. [342, 380, 381, 384]

Importantly, photobleaching is not only a source of error but can also be exploited as an additional source of information. Controlled photobleaching experiments can be used to calibrate molecular brightness, determine labelling stoichiometry, or validate oligomerisation models by observing how brightness distributions evolve as fluorophores are progressively lost. [382, 385]

In the specific case of YO-PRO-1–DNA complexes, both the decay of fluorescence intensity and the evolution of molecular brightness must be interpreted together. Photobleaching reduces the number of emissive complexes within the focal volume, while concurrent changes in brightness reflect alterations in the composition and photophysical state of the remaining population. A joint analysis of these two observables therefore provides a more complete and quantitatively reliable link between photobleaching kinetics and molecular counting in fluorescence fluctuation experiments.

Quantification of Photobleached Complexes in the Focal Volume

Quantitative conversion of fluorescence decay into the number of photobleached complexes requires an explicit relationship between measured intensity, molecular brightness, and the number of fluorescent species within the observation volume. Under ideal conditions — namely, constant per-molecule brightness and linear detection — the total fluorescence signal is proportional to the number of unbleached, fluorescently competent complexes. [342, 372, 373, 377] In this regime, the time-dependent decrease in fluorescence intensity can be directly interpreted as the cumulative loss of active complexes, allowing photobleaching curves to serve as a proxy for molecular counting. [342, 374, 375]

In practice, this proportionality is often only approximately valid. Photobleaching does not simply reduce the number of molecules; it can also alter the apparent molecular brightness of the remaining population. For example, partial bleaching of multi-labelled complexes, changes in photophysical state distributions, or excitation-dependent effects can modify the per-molecule photon output over time. As a result, fluorescence decay alone cannot be unambiguously mapped onto molecule number without additional constraints. In fluorescence correlation spectroscopy, this leads to biased estimates of both particle number and brightness unless bleaching kinetics are explicitly incorporated into the analysis framework. [271, 311, 338, 342, 380, 381, 382, 383, 384, 385]

A robust strategy is therefore to combine photobleaching kinetics with molecular brightness analysis, treating intensity and brightness as coupled observables. By independently tracking the evolution of total fluorescence and per-molecule brightness, it becomes possible to disentangle particle loss from changes in emission efficiency. This joint analysis enables more accurate estimation of the number of remaining complexes and, by difference, the number of photobleached species within the focal volume.

The statistical interpretation of such measurements must also account for noise and heterogeneity. Deviations from single-exponential bleaching behaviour are

common and arise from spatially varying excitation profiles, heterogeneous local environments, reversible dark states, and complex photophysical pathways. [277, 366, 369, 370, 371, 372, 373] These effects lead to multi-exponential or non-exponential decay curves, which cannot be described by a single rate constant and require more sophisticated kinetic or distribution-based models. Moreover, stochastic fluctuations in photon detection, especially at low molecule numbers, introduce additional uncertainty that must be mitigated through temporal averaging or ensemble measurements.

In immobilised or spatially confined systems, these challenges are further amplified because molecular exchange with the surroundings is limited, preventing replenishment of unbleached fluorophores. Consequently, local heterogeneity in binding, environment, or bleaching pathways can strongly influence the observed decay. Careful modelling and, where possible, independent calibration of molecular brightness are therefore essential to ensure that fluorescence decay curves yield reliable quantitative information about the number of photobleached complexes.

Experimental and Instrumental Considerations

Photobleaching kinetics are highly sensitive to experimental conditions, and their quantitative interpretation requires careful control of both chemical environment and excitation parameters. The bleaching rate is not governed by a single intrinsic constant but emerges from the interplay between excitation intensity, oxygen availability, dye concentration, and the local physicochemical environment. Increasing excitation power enhances the population of excited singlet and triplet states, thereby accelerating photochemical reactions, while oxygen concentration modulates reactive oxygen species pathways that often dominate bleaching. At the same time, the surrounding matrix—such as solvent composition or gel structure—affects diffusion, triplet lifetimes, and local reactivity, leading to system-specific bleaching behaviour. Because of this complexity, reliable quantitative measurements generally require empirical calibration under the exact experimental conditions used. [349, 362, 363, 365, 366, 367, 386, 387]

In the context of fluorescence correlation spectroscopy and molecular brightness analysis, these effects become particularly critical. Photobleaching alters not only the number of emitting molecules in the observation volume but also their apparent molecular brightness, especially in confined volumes or under high excitation. This dual effect introduces systematic bias into both autocorrelation amplitudes and brightness-derived parameters if bleaching is treated as negligible. As a consequence, experimental strategies must either minimise bleaching — by operating in the low-power linear regime, maintaining sufficient exchange with unbleached molecules, or using scanning-based approaches — or explicitly incorporate bleaching kinetics into the analysis model. [271, 311, 338, 342, 380, 381, 382, 383, 384, 385]

Instrumental factors further influence the accuracy of photobleaching and brightness measurements. Stable excitation intensity is essential, as fluctuations in laser output can mimic or distort bleaching kinetics. Detector performance, including quantum efficiency, linearity, and dark noise, directly affects the measured photon count rate and, in turn, the inferred molecular brightness. In addition, a precise definition of the observation volume and rigorous background correction are required to ensure that changes in fluorescence intensity reflect true mole-

cular processes rather than optical or electronic artefacts. Improper background subtraction, detector saturation, or non-linear response can lead to systematic errors in both decay curves and brightness estimates, ultimately compromising the reliability of molecular counting.

Taken together, accurate quantification of photobleaching in fluorescence fluctuation experiments depends on tightly controlled excitation conditions, well-characterised detection systems, and careful consideration of environmental factors. Only under these conditions can fluorescence decay and brightness measurements be reliably interpreted in terms of underlying molecular processes.

Context within Photobleaching-Based Techniques

Continuous photobleaching is part of a broader class of fluorescence methods that exploit irreversible or controlled signal loss to extract quantitative information about molecular populations, kinetics, or stoichiometry. In this approach, the gradual decay of fluorescence under steady illumination is interpreted as the cumulative loss of active fluorophores or fluorescent complexes. This interpretation typically relies on simplifying assumptions such as first-order bleaching kinetics, constant molecular brightness, and spatially uniform excitation conditions. When these conditions are reasonably satisfied, fluorescence decay curves can be directly related to the number of remaining emissive species over time. [342, 366, 370, 372, 373, 376, 377, 378, 379, 380]

Within the landscape of photobleaching-based techniques, continuous photobleaching occupies a distinct niche. Unlike fluorescence recovery after photobleaching (FRAP), which probes molecular mobility through fluorescence recovery following a localised bleaching event, continuous photobleaching does not involve recovery and instead focuses on irreversible signal loss under constant illumination. Stepwise single-molecule photobleaching, in contrast, enables direct counting of fluorophores within individual complexes by resolving discrete intensity steps, but it requires low background noise and sufficiently stable emission to distinguish individual bleaching events — conditions that are not always achievable in complex or heterogeneous systems.

Continuous photobleaching offers a complementary strategy by averaging over many molecules within a defined observation volume. Compared with bulk photobleaching assays, which provide only ensemble-averaged information without spatial resolution, confocal implementations retain spatial confinement while maintaining statistical robustness. This makes the method particularly suitable for systems where molecular exchange is limited or absent.

For immobilised DNA systems, this approach is especially advantageous. Because diffusion into and out of the focal volume is suppressed, the observed fluorescence decay reflects intrinsic photobleaching and binding-related processes rather than transport dynamics. As a result, continuous photobleaching provides a practical balance between ensemble-level averaging and localised measurement, enabling quantitative analysis of dye–DNA complexes under well-controlled experimental conditions while remaining compatible with complementary techniques such as fluorescence correlation spectroscopy and molecular brightness analysis.

2 Materials and methods

2.1 Materials

2.1.1 Buffers

Phosphate Saline Buffer, PBS

Phosphate-buffered saline (PBS) was prepared according to the manufacturer's instructions (Sigma-Aldrich) with a final pH of 7.4. The buffer composition included 0.01 mM phosphate, 0.0027 M potassium chloride, and 0.137 M sodium chloride. To prevent the probe from attaching to the glass surface during experiments, 0.002% Tween 20 (Sigma-Aldrich) was added to the solution, ensuring smooth, consistent measurements.

2.1.2 Nucleic acids

Double-stranded 69 bp oligonucleotide

For the brightness experiments, a 69-base-pair (bp) double-stranded oligonucleotide was synthesised by Integrated DNA Technologies and purchased lyophilised. The DNA was suspended in a standard Tris-EDTA buffer (Sigma-Aldrich) at 70 μ M and stored at -20°C. Before each measurement, the oligonucleotide was diluted to working concentrations ranging from 20 pM to 500 nM. Phosphate-buffered saline (PBS) (Sigma-Aldrich) with 0.002% Tween 20 surfactant (Sigma-Aldrich) was used as the solvent for dilution, as described in the Materials: Buffers section. The sequence of the oligonucleotide:

5' GAT ACG AGC ATC GTG TAG GCA TCG TAG GTA ATA CGG (...)
3' CTA TGC TCG TAG CAC ATC CGT AGC ATC CAT TAT GCC (...)

(...) ATG TGC TAG CTT ATT GAA TTC AGA GAT CTA TGC 3'
(...) TAC ACG ATC GAA TAA CTT AAG TCT CTA GAT ACG 5'

Phage λ DNA

In the continuous fluorescence photobleaching experiments, phage λ DNA (ThermoFisher) was used. The DNA (9.52 μ M) was stored in a standard Tris-EDTA buffer (Sigma-Aldrich) at -20°C. Prior to the experiments, the DNA was diluted to the required concentrations for continuous fluorescence photobleaching and agarose gel preparation.

tRNA

I used tRNA (Thermo Fisher Scientific) to determine the equilibrium constant for the reaction between tRNA and the YO-PRO-1 dye. The lyophilised powder was resuspended in Tris-EDTA buffer at 1 mM. Further dilution was performed in a 10x step using PBS buffer containing 0.002% Tween20 surfactant to prevent sample adhesion to the glass surface during the experiments.

YO-PRO-1 targets

I used for YO-PRO-1 target determination single-stranded DNA (IBA GmbH; TAG CAC ATC CGT A sequence), Human Serum Albumin (Sigma-Aldrich), hairpins (IBA GmbH; TTT AAT TAA TCG TTG TTA AAA AAA TTT TAA CCC CTT AAA ATT TTT TTA ACA ACG ATT AAT TAA A sequence), RNA control 250 (Thermo Fisher Scientific, 2000 nt RNA transcript; the sequence is proprietary), the total pool of RNA (Thermo Fisher Scientific, catalogue number: 4307281) and tRNA. All nucleic acids were dissolved in PBS buffer supplemented with 0.002% Tween20 for measurements.

2.1.3 Dyes and anthracyclines

Cyanine dyes

YO-PRO-1 and YOYO-1 dyes (Invitrogen) were purchased at a concentration of 1 mM. The stock solutions were prepared by diluting the purchased stocks with PBS containing Tween 20, as described in the Materials: Buffers section. The dyes were diluted stepwise, with each dilution step being 10 times. The working solutions were then prepared from the stock, ensuring the dilution factor did not exceed 10.

Anthracyclines

Anthracyclines: doxorubicin (DOX, Santa Cruz Biotechnology Inc), daunorubicin (DAU, Sigma-Aldrich), idarubicin (IDA, Sigma-Aldrich) and epirubicin (EPI, Sigma-Aldrich) were purchased as a solid hydrochloride. The stock solutions were prepared in Milli-Q water to achieve a concentration of 1 mM. For subsequent experiments, 10x dilutions were performed after 30 minutes of sonication to ensure complete dissolution and uniformity of the solution.

2.2 Methods

2.2.1 FCS set-up

The Fluorescence Correlation Spectroscopy (FCS) setup consisted of an integrated Nikon C1 confocal microscope and a time-correlated single-photon counting (TCSPC) data-acquisition system (PicoHarp 300, PicoQuant). All measurements were performed using a Nikon PlanApo 60x water-immersion objective (NA = 1.2). Excitation was provided by a laser diode emitting picosecond pulses at a wavelength of 485 ± 3 nm. The emitted fluorescence was captured by a Single Photon Avalanche Diode (SPAD) detector from PerkinElmer Optoelectronics. Fluorescence emission was filtered through a 488 nm long-pass filter (Chroma) positioned in the optical path before reaching the detector. Data acquisition was performed using Symphotime 64 software (PicoQuant). Temperature control was maintained using the Okolab Cage Incubation System, ensuring precise temperature stability with an accuracy of 0.5°C .

2.2.2 FCS measurements

The temperature was maintained at $25 \pm 0.5^\circ\text{C}$ (or $36 \pm 0.5^\circ\text{C}$) throughout the experiments. Laser powers of 30, 42, 47, or 50 μW (depending on the experiments) were utilised, measured before the objective using a power meter (PM400,

Thorlabs). At such a low laser power, there was no photobleaching of molecules, and the contribution of the triplet state to the acquired signal was minimised. Before conducting the experiments, the dimensions of the confocal volume were determined through calibration using rhodamine 110 (Sigma-Aldrich) dissolved in PBS. Glass-bottom containers (μ -Slide 8 Well Glass Bottom, Ibidi) were used for all measurements, with FCS measurements taken 10 μm above the glass cover. The acquired data were analysed using self-written Python scripts.

2.2.3 Calibration

Before conducting measurements, I calibrated the FCS setup by employing a fluorescent dye with a known size and diffusion coefficient. Specifically, I utilised Rhodamine 110 (with diffusion coefficients of 470 $\mu\text{m}^2/\text{s}$ at 25°C and 580 $\mu\text{m}^2/\text{s}$ at 36°C; Sigma-Aldrich) at the temperature used during measurements (25 or 36°C). This allowed me to determine the semi-minor axis of the confocal volume (ω) and the effective volume (V_{eff}). I conducted 24 individual dye measurements and then fitted the data using a one-component autocorrelation function, $G(t)$ (as described in Equation 1).

$$G(t) = \left(\frac{1}{N}\right) \cdot \left(1 + \frac{T}{1+T} \cdot \left(\frac{1}{\exp\frac{t}{\tau_{\text{triplet}}}}\right)\right) \cdot \left(\frac{1}{(1+\frac{t}{\tau})}\right) \cdot \left(\frac{1}{\sqrt{1 + (\frac{t}{\tau}) \cdot \frac{1}{\chi^2}}}\right) \quad (1)$$

Where N is the number of molecules in the focal volume, T is the fraction of triplet states, τ_{triplet} is the triplet state lifetime, τ is the dye's time of flight through the focal volume, and χ is the aspect ratio of the focal volume.

The autocorrelation functions were exported to a .csv file and analysed using a self-written Python script. The analysis involved employing an appropriate diffusion model based on Equation 1, utilising a non-linear least squares fitting method (Fig. 18 and Fig. 19). Parameters N , T , τ_{triplet} , τ and χ were free parameters during the fitting process.

The obtained values from the measurements were averaged, and the semi-minor axis of the confocal volume, ω , was calculated using the formula:

$$\omega = \sqrt{4\tau D} \quad (2)$$

Then, the determined value of ω was used to calculate the effective volume of the confocal focus, V_{eff} :

$$V_{\text{eff}} = \pi^{3/2} \chi \omega^3 \quad (3)$$

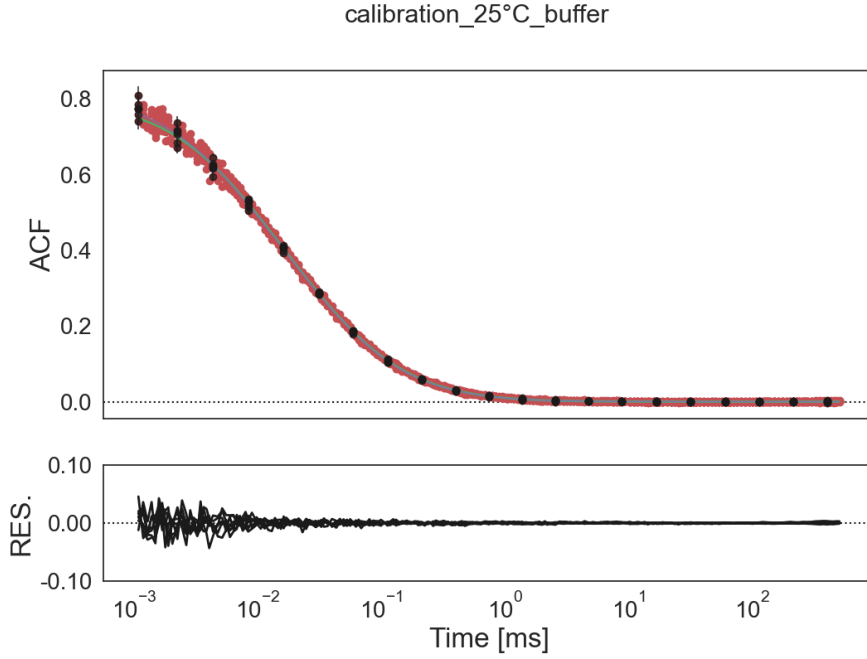


Fig. 18: The plot illustrates an example of FCS autocorrelation curves acquired during calibration (in a buffer environment, 25°C). Each autocorrelation curve was measured over 20 seconds. The data were fitted using a one-component diffusion model that accounts for the contribution from the triplet state (as per Equation 1). Experimental data points are depicted in red, while the green line represents the autocorrelation function resulting from the fitting process. In the plot, there are 6 separate autocorrelation curves, with the fitting curve. Additionally, the bottom panel displays the residual curve, which denotes the disparity between the experimental and fitted data points.

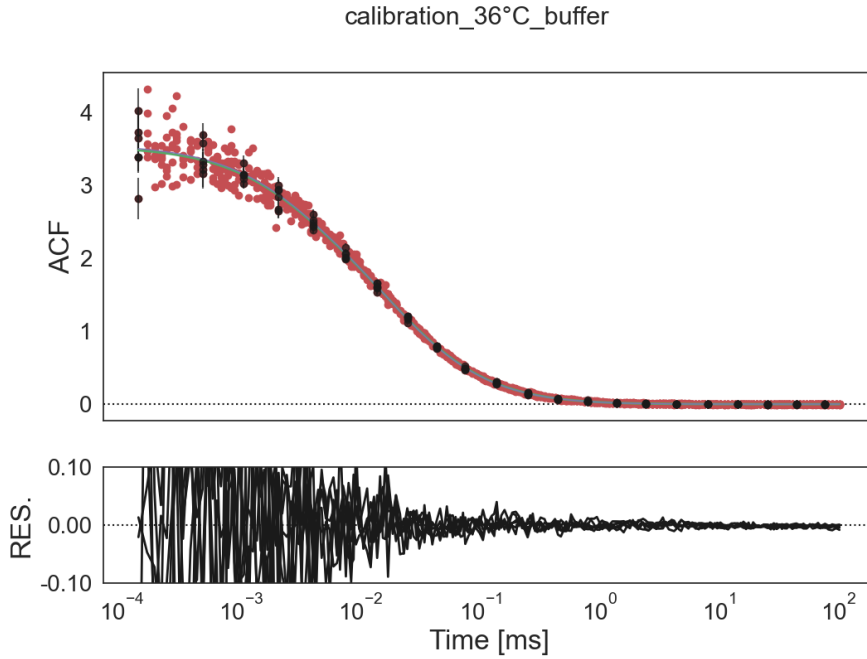


Fig. 19: The plot illustrates an example of FCS autocorrelation curves acquired during calibration (in a buffer environment, 36°C). Each autocorrelation curve was measured over 20 seconds. The data were fitted using a one-component diffusion model that accounts for the contribution from the triplet state (as per Equation 1). Experimental data points are depicted in red, while the green line represents the autocorrelation function resulting from the fitting process. In the plot, there are 6 separate autocorrelation curves, with the fitting curve. Additionally, the bottom panel displays the residual curve, which denotes the disparity between the experimental and fitted data points.

2.2.4 Brightness method samples

The DNA used in brightness method samples was prepared as follows: DNA in aliquots (diluted in TE, lyophilised DNA purchased from the manufacturer) was diluted in PBS buffer in stocks, with concentrations 1 μ M, 100 nM, 10 nM and 1 nM. The DNA concentration of the sample was determined by further diluting the respective stock.

The tRNA samples used in the molecular brightness method were prepared following a systematic dilution process. Initially, lyophilised tRNA obtained from the manufacturer was reconstituted in TE buffer. The stock solutions were then prepared in PBS buffer at concentrations of 1 μ M, 100 nM, 10 nM and 1 nM. The desired tRNA concentration for each experiment was achieved by further diluting the appropriate stock solution, ensuring accurate and reproducible sample preparation.

The dyes and anthracyclines were prepared in stocks in PBS buffers, with the addition of 0.002% Tween20 surfactant. The aliquots were diluted no more than 10x. Before dilution, anthracyclines were sonicated for 30 minutes to prevent aggregation. Fluorophores were diluted 2-fold from the final stock before sample preparation.

2.2.5 Brightness model

In the brightness analysis method, I observe the variation in molecular brightness (MB) upon the occurrence of a reaction, such as complex formation. MB is defined as the number of photons (N_{photons}) emitted by a molecule within a given time interval (t). Various factors, including the properties of the fluorescent molecule, laser power, wavelength, and detector sensitivity, influence its value [229]. The expression for MB is as follows:

$$\text{MB} = \frac{1}{t} \frac{N_{\text{photons}}}{N_{\text{molecules}}} \quad (4)$$

where $N_{\text{molecules}}$ represents the number of molecules in the focal volume. The count rate (I) is the number of emitted photons per unit time.

$$I = \frac{N_{\text{photons}}}{t} \quad (5)$$

Using equation 5, I rewritten MB as:

$$\text{MB} = \frac{I}{N_{\text{molecules}}} \quad (6)$$

I expressed the number of molecules in terms of concentrations, utilising the Avogadro constant (N_A) and the effective volume of the focal volume (V_{eff}):

$$N_{\text{molecules}} = C \cdot N_A \cdot V_{\text{eff}} \quad (7)$$

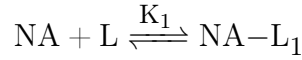
Combining equations 6 and 7, the count rate equals:

$$I = MB \cdot C \cdot N_A \cdot V_{\text{eff}} \quad (8)$$

The intensity (I_0) of the free dye (or anthracycline), characterised by its molecular brightness (α), is expressed as follows:

$$I_0 = \alpha \cdot C_L \cdot N_A \cdot V_{\text{eff}} \quad (9)$$

where C_L is the free dye concentration (or anthracycline) concentration. For the YO-PRO-1 and YOYO-1 dyes, the MB of the free dye was calculated out of the statistical number of molecules, as the dyes themselves are non-fluorescent in an unbound state.



Complex formation occurs upon adding DNA (or tRNA; nucleic acid - NA). The chemical equation describes the following cases:

- a) the mono-intercalation of YOYO-1 into DNA,
- b) the reaction of YO-PRO-1 and DNA (or tRNA),
- c) the intercalation of anthracyclines into DNA.

The equilibrium constant, K_1 , for that reaction is given by:

$$K_1 = \frac{C_{NA-L_1}^{\text{eq}}}{C_{NA}^{\text{eq}} C_L^{\text{eq}}} = \frac{C_{NA-L_1}^{\text{eq}}}{(C_{NA}^0 - C_{NA-L_1}^{\text{eq}})(C_L^0 - C_{NA-L_1}^{\text{eq}})} \quad (10)$$

where $C_{NA-L_1}^{\text{eq}}$ is the first step of the reaction (or the only step in case of YO-PRO-1) in the equilibrium state, C_L^0 and C_{NA}^0 are the initial concentrations of the dye (or anthracycline) and the DNA (or tRNA), respectively. I devised a quadratic equation with the concentration of the first step of the reaction complex in the equilibrium state as the variable:

$$K_1(C_{NA-L_1}^{\text{eq}})^2 - C_{NA-L_1}^{\text{eq}}(K_1 C_{NA}^0 + K_1 C_L^0 + 1) + K_1 C_{NA}^0 C_L^0 = 0 \quad (11)$$

I solved Equation 11, deriving the relation describing the concentration of the first step of the reaction complex in the equilibrium state.

$$C_{NA-L_1}^{\text{eq}} = \frac{1}{2} \cdot (C_{NA}^0 + C_L^0 + \frac{1}{K_1} - \sqrt{(-C_{NA}^0 - C_L^0 - \frac{1}{K_1})^2 - 4C_{NA}^0 C_L^0}) \quad (12)$$

I described intensity (I_1) for the probe with the first step of the reaction complex as:

$$I_1 = V_{\text{eff}} \cdot N_A \cdot (\alpha \cdot C_L^{\text{eq}} + \gamma_1 \cdot C_{NA-L_1}^{\text{eq}}) \quad (13)$$

where γ_1 represents the molecular brightness of the first step of the formed complex. I combined Equation 10 and Equation 13 to establish a relationship between intensity, initial concentrations, and the equilibrium constant K_1 :

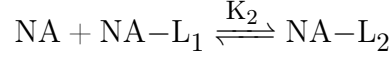
$$I_1 = V_{\text{eff}} \cdot N_A \cdot \alpha \cdot ((C_L^0 - C_{NA-L_1}^{\text{eq}}) + \frac{\gamma_1}{\alpha} \cdot K_1 \cdot (C_{NA}^0 - C_{NA-L_1}^{\text{eq}}) \cdot (C_L^0 - C_{NA-L_1}^{\text{eq}})) \quad (14)$$

I incorporated the size of the binding site into the model by representing nucleic acid (NA) concentration as the number of binding sites:

$$I_1 = V_{\text{eff}} \cdot N_A \cdot \alpha \cdot ((C_L^0 - C_{\text{NA-L}_1}^{\text{eq}}) + \frac{\gamma_1}{\alpha} \cdot K_1 \cdot (\frac{C_{\text{NA}}^0}{n} - C_{\text{NA-L}_1}^{\text{eq}}) \cdot (C_L^0 - C_{\text{NA-L}_1}^{\text{eq}})) \quad (15)$$

where n is the size of the binding site.

I described the second step reaction (bis-intercalation in the case of YOYO-1 or attachment of the additional anthracycline molecule to the formed complex) with the equation:



where DNA-L_2 represents the second step reaction complex. The equilibrium constant K_2 for this reaction is given by:

$$K_2 = \frac{C_{\text{NA-L}_2}^{\text{eq}}}{C_{\text{NA}}^{\text{eq}} C_{\text{NA-L}_1}^{\text{eq}}} \quad (16)$$

I determined the concentration of bis-intercalated complex in equilibrium state from Eqn 16:

$$C_{\text{DNA-L}_2}^{\text{eq}} = K_2 \cdot (C_{\text{NA}}^0 - C_{\text{NA-L}_1}^{\text{eq}}) \cdot C_{\text{NA-L}_1}^{\text{eq}} \quad (17)$$

Intensity (I_2), including both reaction steps, is given by:

$$I_2 = V_{\text{eff}} \cdot N_A \cdot (\alpha \cdot C_L^{\text{eq}} + \gamma_1 \cdot C_{\text{NA-L}_1}^{\text{eq}} + \gamma_2 \cdot C_{\text{NA-L}_2}^{\text{eq}}) \quad (18)$$

Combining Equations 14, 17, and 18, I derived the fitting equation for the second step reaction equilibrium constant K_2 :

$$I_2 = V_{\text{eff}} \cdot N_A \cdot \alpha \cdot ((C_L^0 - C_{\text{NA-L}_1}^{\text{eq}}) + (\frac{\gamma_1}{\alpha} \cdot K_1 \cdot (C_{\text{NA}}^0 - C_{\text{DNA-L}_1}^{\text{eq}}) \cdot (C_L^0 - C_{\text{NA-L}_1}^{\text{eq}})) + (\frac{\gamma_2}{\alpha} \cdot K_2 \cdot (C_{\text{NA}}^0 - C_{\text{NA-L}_1}^{\text{eq}}) \cdot C_{\text{NA-L}_1}^{\text{eq}})) \quad (19)$$

In the final model for fitting K_2 , NA concentration was characterised as the concentration of binding sites:

$$I_2 = V_{\text{eff}} \cdot N_A \cdot \alpha \cdot ((C_L^0 - C_{\text{NA-L}_1}^{\text{eq}}) + (\frac{\gamma_1}{\alpha} \cdot K_1 \cdot (\frac{C_{\text{NA}}^0}{n} - C_{\text{NA-L}_1}^{\text{eq}}) \cdot (C_L^0 - C_{\text{NA-L}_1}^{\text{eq}})) + (\frac{\gamma_2}{\alpha} \cdot K_2 \cdot (\frac{C_{\text{NA}}^0}{n} - C_{\text{NA-L}_1}^{\text{eq}}) \cdot C_{\text{NA-L}_1}^{\text{eq}})) \quad (20)$$

2.2.6 Polarisation experiments

Sample preparation

Samples for immobilised DNA experiments were prepared using Phage λ DNA, YO-PRO-1 dye, and PBS buffer supplemented with Tween 20 surfactant. For calibration purposes, Rhodamine 110 was used.

To accurately estimate molecular brightness, one sample was prepared in a buffer containing 0.5 nM Phage λ DNA and 100 μ M YO-PRO-1, in a total volume of 300 μ L.

For DNA-YO-PRO-1 samples that were immobilised in a gel, a smaller 100 μ L volume was used. In the gel, the final concentrations of DNA and YO-PRO-1 were 30 pM and 100 μ M, respectively.

The DNA and YO-PRO-1 dye samples were incubated for 2 hours at 25°C or overnight at 36°C to allow for equilibrium in the complex formation reaction.

Gel preparation

DNA was immobilised in a 5% agarose gel. The gel was prepared by dissolving Low Melting Point Agarose (Invitrogen) in PBS containing Tween20. The solution was vortexed for 5 seconds and then incubated in a thermoshaker at 70°C and 300 rpm for 1 hour to ensure proper dissolution and homogenization of the gel.

In the determination study of the gel content and concentration, gelatin (Sigma-Aldrich) was also used.

DNA immobilisation

After incubating the DNA-YO-PRO-1 samples and the gel, I prepared the immobilised DNA samples. The gels were vortexed for 5 seconds, followed by vortexing of the samples. The samples were then added to the gels, vortexed again, and pipetted into the wells of an 8-well plate (Ibidi). To prevent oxygen exposure, the plate was sealed with parafilm (a thin film of pure paraffin) and incubated at 4°C for 12 hours to ensure proper gelation.

Confocal set-up

To visualise the immobilised λ -DNA-YO-PRO-1 complexes, I employed a Nikon A1 confocal microscope controlled by NIS Elements software (Nikon). The YO-PRO-1 dye, which exhibits maximum fluorescence emission at $\lambda_{\text{max}} = 491$ nm, was excited using a 485 nm laser. This setup enabled efficient dye excitation and accurate imaging of the immobilised complexes.

Measurement parameters

Measurements were performed by coupling FCS, fluorescence intensity measurements and confocal imaging. The FCS cross-correlation was applied to eliminate detector after-pulsing. For that purpose, two detectors with 488 long-pass filters (Chroma) were used. The polarisation module consisted of a linear polariser followed by a quarter-wave plate that converted the polarisation to circular or linear, together with an additional optical fibre that elongated the optical pathway. The schematic pathway of the signal is shown in Fig. 20.

The set-up was calibrated in both buffer and gel using Rhodamine 110. The temperature was set at 25 (or 36) $\pm$ 0.5°C and controlled using the Okolab Cage Incubation System.

Data analysis

The λ -DNA molecule has a diameter of 491 nm. Therefore, it is wider than the focal volume. To calculate visible, excited-emitting complexes of λ -DNA-YO-PRO-1 complexes, the cylinder approximated the volume of the DNA in the focal volume. The cylinder was characterised by a radius equal to ω - half of the width of the focal volume and a height equal to the diameter of the λ -DNA (Fig. 21). Then, the number of binding sites in the cylindrical volume was calculated and

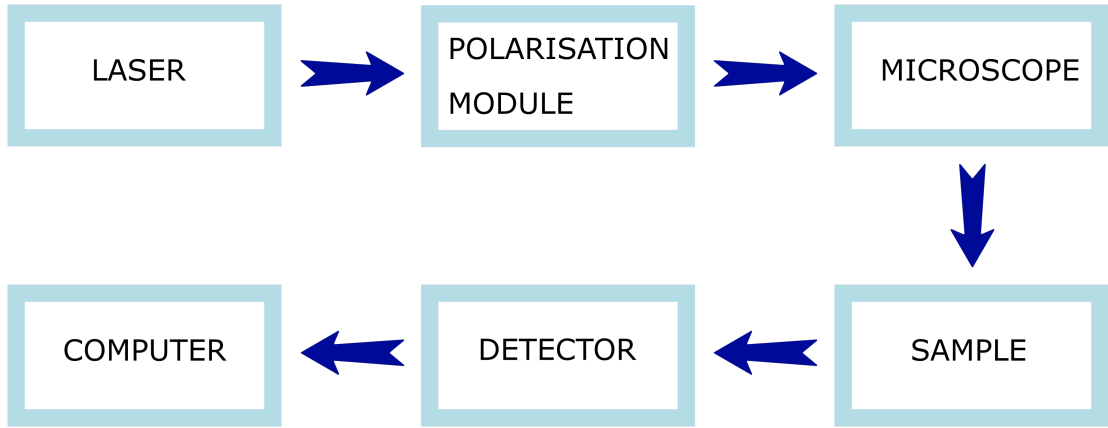


Fig. 20: Scheme of the optical pathway. A laser pulse is polarised in the polarisation module before reaching the sample in the microscope. The fluorescence signal coming from the sample is then detected by two SPAD detectors and transferred to the computer for analysis in the Syphotime program.

used as a maximum of excited-emitting complexes in the focal volume. Then, based on the continuous fluorescence photobleaching measurements, the fraction of excited-emitting complexes was calculated.

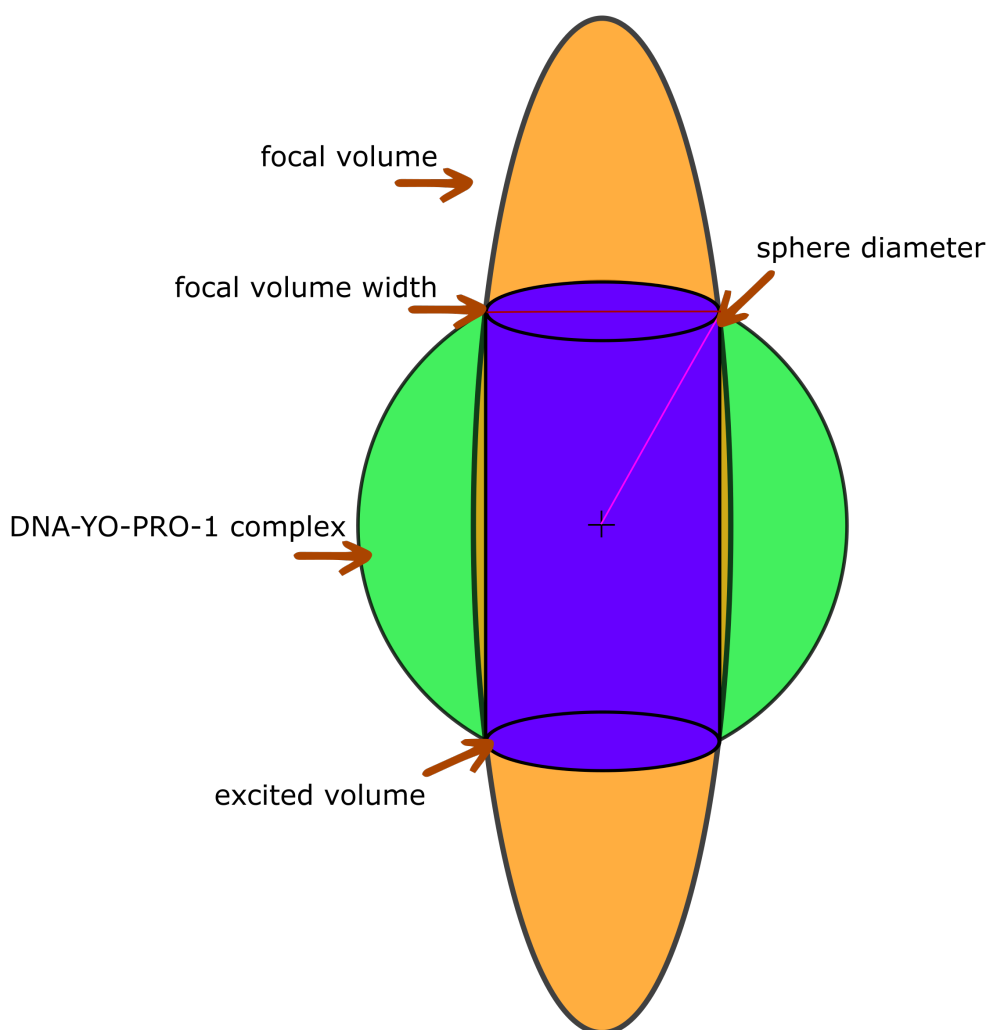


Fig. 21: Scheme of the λ -DNA (green) in focal volume (orange). The cylinder approximation (purple) was used to calculate the DNA volume within the focal volume.

2.2.7 YO-PRO-1 target determination

Cell lysis

HeLa cells, obtained from the American Type Culture Collection (ATCC, Manassas, USA), were cultured as a monolayer under standard conditions (37°C, 5% CO₂). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 1 g L⁻¹ glucose (Institute of Immunology and Experimental Technology, Wrocław, Poland), supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine (Sigma-Aldrich), and 1% (v/v) antibiotic solution comprising streptomycin (10 mg mL⁻¹, Merck) and penicillin (10,000 U mL⁻¹, Sigma-Aldrich). To ensure continuous proliferation, cells were routinely passaged to maintain logarithmic growth. For subculturing, cells were detached using 0.25% Trypsin-EDTA solution (Sigma-Aldrich), and the detachment process was monitored by light microscopy.

After detachment from the culture surface, the cells were collected by centrifugation, and the supernatant was discarded. The resulting cell pellet was lysed using 1 mL of a non-denaturing buffer composed of 10 mM imidazole, 0.5 M sodium chloride, 1% Triton X-100, 0.2 mM sodium orthovanadate, and 0.2 mM phenylmethylsulfonyl fluoride. To generate a lysate with degraded RNA, an identical buffer was prepared and additionally supplemented with 10 mg/mL RNase (Sigma-Aldrich). Cells were incubated in each respective buffer for 15 minutes to complete lysis.

3 Results and discussion

3.1 Equilibrium constant determination

The biological world remains largely unexplored, particularly in biochemistry, where many processes remain enigmatic. Despite decades of research on cellular-level reactions, our understanding is still limited, akin to a single droplet in the vast ocean of knowledge.

In this thesis, I focus on the kinetic analysis of several key reactions: those involving DNA and dyes, DNA and anthracyclines, and tRNA and the YO-PRO-1 dye.

My research focused on studying the interactions between intercalators and nucleic acids, specifically DNA and tRNA, to determine their consecutive equilibrium constants.

Cyanine dyes, such as YO-PRO-1 and its homodimer YOYO-1, are widely used in research as cellular DNA stains for qualitative and quantitative analyses. Despite their frequent application, the mechanisms of action, equilibrium constants, and specific cellular targets of these dyes remain largely unknown.

Nevertheless, conclusions have been drawn from these analyses, often without addressing the underlying uncertainties, leaving many ambiguous findings unresolved.

Circular dichroism studies conducted in the 1960s on YOYO-1 suggested the possibility of an additional interaction mechanism with DNA beyond intercalation. However, this hypothesis has not been extensively explored.

Despite the lack of clarity about its precise mode of interaction with DNA, YOYO-1 remains widely used in quantitative research.

Anthracyclines, a family of antibiotics, are a group of molecules with unclear kinetics and reaction mechanisms. Moreover, in addition to their therapeutic effects, anthracyclines are associated with dose-dependent cardiotoxicity, which remains a significant limitation in their clinical use.

Equilibrium constants should be the essential parameter for cardiotoxicity. While equilibrium constants have been reported in the literature, their values vary widely, and the two-step reaction mechanism has not been thoroughly investigated or characterised. Recent studies have examined the mechanisms of their reactions with DNA, including the determination of equilibrium constants at 25°C [188].

The research mentioned above was conducted in our group, so I decided to repeat the experiments at 36°C. The same equipment allowed me to compare the obtained results well.

Additionally, in my research, I investigated the membrane permeation and cellular targets of YO-PRO-1 as a step toward cell-based measurements. This work led to further analysis of the YO-PRO-1 reaction with tRNA and to the determination of the equilibrium constant.

Based on experimental results, I found that the dye is not only cell-permeant but also does not interact with nuclear DNA. Instead, YO-PRO-1 primarily targets the large and small ribosomal subunits, whole ribosomes, and tRNA [?].

To my knowledge, the interaction between tRNA and YO-PRO-1 has not been previously reported, nor has its equilibrium constant been characterised.

3.1.1 Optimisation of laser power

Initial work with a new dye requires careful determination of the proper, yet sufficient, laser power to avoid photobleaching of the fluorophore during measurements and to obtain accurately high fluorescence intensities, enabling the observation of the probe and the occurring reaction. In addition, selecting the optimal dye concentration is crucial to ensure a strong fluorescence signal without saturating the detector.

The results presented in this chapter concern the use of YO-PRO-1 and YOYO-1 cyanine dyes and the anthracyclines doxorubicin (DOX), daunorubicin (DAU), idarubicin (IDA), and epirubicin (EPI).

The optimal laser power (50 μW) was previously determined using the same equipment [188], so I followed the established framework.

For cyanine dyes, however, I needed to determine the optimal laser power.

Before conducting the experiment, the equipment was calibrated with Rhodamine 110. Next, I prepared samples and measured their fluorescence intensities at increasing laser power (Table 9).

Based on the plot (Fig. 22), I decided to work with YO-PRO-1 dye using 30 μW laser power, and for YOYO-1 dye, the optimal laser power is 50 μW . At intensities below these thresholds, recorded intensities increased linearly, indicating no photobleaching of the samples.

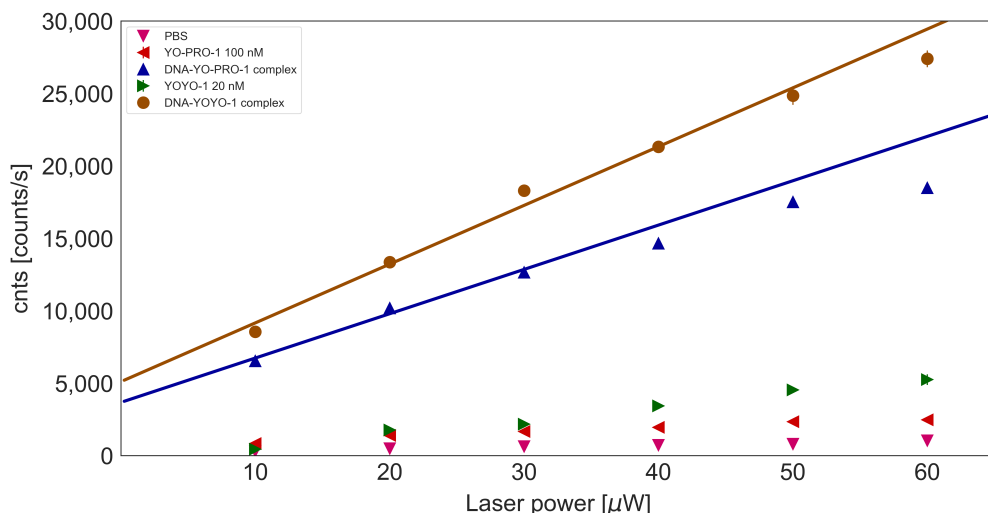


Fig. 22: Collected fluorescence for measured samples upon increased applied laser power. PBS, and YO-PRO-1 and YOYO-1 dyes in a free state, exhibit no fluorescence properties; collected photons are connected with a dispersion of the laser beam on molecules present in the focal volume. The fluorescent complex of DNA (100 nM) and YO-PRO-1 (20 nM) displays linear response in fluorescence intensity upon laser power increase up to 30 μW , while for the DNA (2 nM) and YOYO-1 (20 nM) complex, linear response is observed up to 50 μW . Beyond these thresholds, the complexes undergo photobleaching during measurement.

Table 9: Collected photons per second for measured samples upon increasing laser power. The initial concentrations for the complex formation were 100 nM for DNA and 20 nM for the YO-PRO-1 dye reaction, and 20 nM for YOYO-1 dye in the reaction with 2 nM DNA. Reactions were performed at 25°C, in equilibrium state.

Laser power [μ W]	Countrate [kcounts $\cdot$ s $^{-1}$]				
	PBS	YO-PRO-1 100 nM	DNA-YO-PRO-1	YOYO-1 20 nM	DNA-YOYO-1
10	0.4	0.8	6.5	0.4	8.6
20	0.5	1.4	10.2	1.8	13.4
30	0.6	1.7	12.7	2.2	18.3
40	0.7	2.0	14.7	3.4	21.3
50	0.8	2.3	17.5	4.5	24.9
60	1.0	2.5	18.5	5.2	27.4

3.1.2 Mechanisms of studied reactions

The goal, after the determination of optimal laser power, was to study the mechanisms of the reactions. I divided this chapter into sections on cyanine dyes reactions and anthracyclines reactions.

Cyanine dyes: YO-PRO-1 and YOYO-1

In my PhD project, I studied reactions of YOYO-1 and YO-PRO-1 dyes with 69 bp DNA, at two temperatures, 25°C and 36°C. Additionally, I investigated the reaction between YO-PRO-1 dye and tRNA at 36°C, as tRNA is a cellular target of YO-PRO-1.

Both dyes are intercalators that stain double-stranded DNA. Moreover, YOYO-1 is a homodimer of YO-PRO-1 dye, which might indicate the presence of an additional mode of reaction.

I performed a series of titration experiments for all studied reactions at both temperatures. In every experiment, I use a constant dye concentration and a range of DNA concentrations. I collected and recorded fluorescence intensities for all the reactions in separate tables (listed below). The intensities were collected for all individual samples from all data sets. For better visualisation, I plotted the averaged intensities versus DNA concentrations (Fig. 23).

Reactions performed at 25°C:

70 nM YO-PRO-1 dye and the range of DNA concentrations 0.5-200 nM (Table 10);
20 nM YOYO-1 dye and the range of DNA concentrations 0.02-50 nM (Table 11).

Reactions performed at 36°C:

40 nM YO-PRO-1 dye and the range of DNA concentrations 1-100 nM (Table 12);
20 nM YOYO-1 dye and the range of DNA concentrations 1-50 nM (Table 13);
40 nM YO-PRO-1 dye and the range of tRNA concentrations 1-200 nM (Table 14).

Based on the data shown in Fig. 23, the decrease in fluorescence intensity observed at higher temperatures is prominent, highlighting the significant influence of temperature on dyes' behaviour. Elevated temperatures appear to affect not only the intrinsic fluorescence properties of the dyes but also their binding affinities and interaction dynamics with nucleic acids, leading to overall weaker emission.

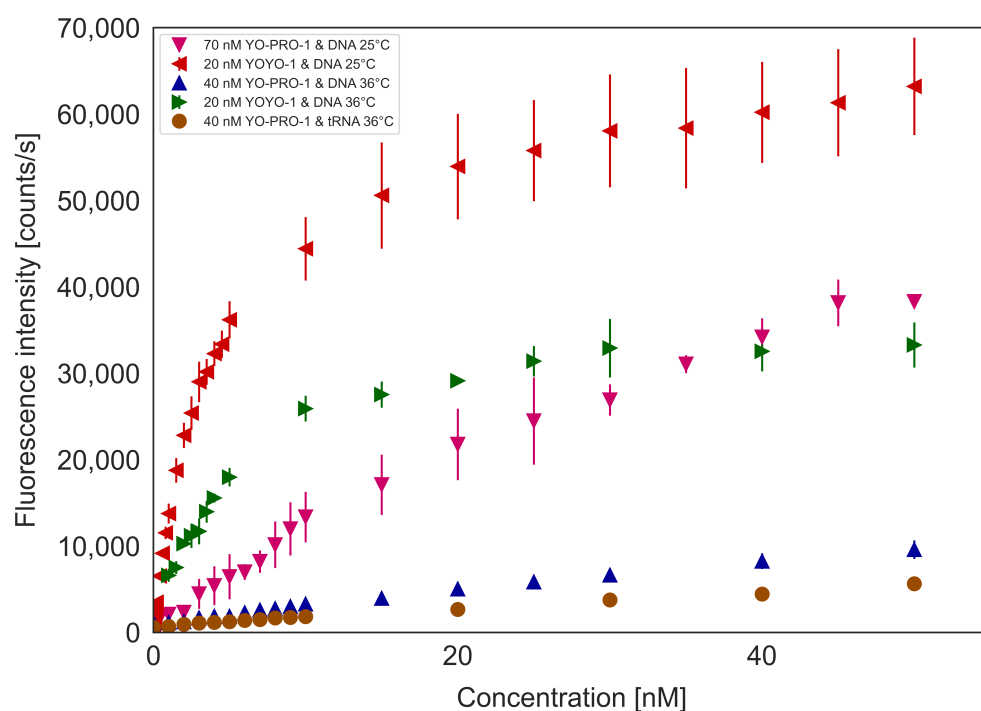


Fig. 23: Relation of the fluorescence intensity and DNA (or tRNA) concentration added to the YO-PRO-1 or YOYO-1 dye. The fluorescence is coming from the excited complexes formed between nucleic acid and the dye. The data were presented in the range 0-50 nM of nucleic acids for better visualisation. Data points are represented with error bars. The pink points ($\blacktriangledown$) correspond to 70 nM YO-PRO-1 bound to DNA at 25°C, the red points ($\blacktriangleleft$) to 20 nM YOYO-1 bound to DNA at 25°C, the blue points ($\blacktriangleright$) to 40 nM YO-PRO-1 bound to DNA at 36°C, the green ($\blacktriangleright$) points to 20 nM YOYO-1 bound to DNA at 36°C, and the brown points ($\bullet$) to 40 nM YO-PRO-1 bound to tRNA at 36°C. Along the x-axis, DNA (or tRNA) concentration ranges from 0 to 50 nM, while the y-axis shows fluorescence intensities up to 70,000 counts/s.

Table 10: Measured samples and collected photons per second of DNA-YO-PRO-1 complex, for all sets of titration experiments. The reaction was performed at 25°C. The initial concentration of YO-PRO-1 dye was 70 nM, and was kept constant. The DNA concentration was varied from 0.5 to 200 nM. " - "marks the incorrectly prepared sample, resulting in a non-physical value of the collected fluorescence intensity.

DNA concentration [nM]	Count rate [photons · s ⁻¹]						mean value [kcounts]
	set 1	set 2	set 3	set 4	set 5	set 6	
0	1549	977	-	971	969	687	1.0 ± 0.3
0.5	1656	1570	-	-	-	2652	2.0 ± 0.5
1	2213	1670	2902	1684	2055	-	2.1 ± 0.5
2	-	2268	-	1917	2004	3134	2.3 ± 0.5
3	3737	7790	-	2702	4135	4014	4.5 ± 1.7
4	4628	10248	4002	3366	5382	4907	5.4 ± 2.3
5	4758	12156	4882	4833	6176	5926	6.5 ± 2.6
6	5867	5394	7010	6192	7569	8363	7.0 ± 0.9
7	6998	6219	8052	7432	-	10368	8.2 ± 1.3
8	7696	15750	9264	8063	9300	10836	10.2 ± 2.7
9	-	16718	-	8520	10207	12532	12.0 ± 3.1
10	11249	17887	-	10359	-	13957	13.4 ± 2.9
15	15140	23683	14799	12897	17444	18617	17.1 ± 3.5
20	-	28282	17966	16727	22353	23530	21.8 ± 4.1
25	20676	32927	22375	19086	-	27337	24.5 ± 5.1
30	24540	-	25211	27466	27786	29558	26.9 ± 1.8
35	31341	-	-	31179	29398	32285	31.1 ± 1.0
40	37809	34798	30454	34037	33164	34786	34.2 ± 2.2
45	42527	37190	-	35181	-	37728	38.2 ± 2.7
50	-	39098	-	38261	37392	38404	38.3 ± 0.6
55	50917	-	39799	38678	41742	40589	42.3 ± 4.4
60	51172	43866	43614	-	44292	42443	45.1 ± 3.1
65	61255	45861	45522	43183	44587	44594	47.5 ± 6.2
70	-	46096	-	43701	47658	47591	46.3 ± 1.6
75	-	47327	49675	49011	49540	49152	48.9 ± 0.8
80	53268	48063	52081	52034	49934	50843	51.0 ± 1.7
85	54011	48783	53050	53596	50529	52381	52.1 ± 1.8
90	-	52349	-	-	53682	52987	53.0 ± 0.5
95	-	54169	55051	58226	54204	55019	55.3 ± 1.5
100	-	55104	57021	60258	54373	56808	56.7 ± 2.0
120	54485	57728	57905	61023	59593	60346	58.5 ± 2.2
140	72401	60402	-	-	60203	63005	64.0 ± 5.0
160	-	63950	63858	72593	64921	64159	65.9 ± 3.4
180	74210	65993	67060	-	67603	68252	68.6 ± 2.9
200	77775	-	69173	77732	-	72299	74.2 ± 3.7
500	93131	95838	101822	95429	91489	90727	94.7 ± 3.7

Table 11: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 25°C, in equilibrium state. The initial concentration of YOYO-1 dye was 20 nM. The dye concentration was kept constant. The DNA concentration ranges from 0.02 to 50 nM. " - "marks for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Count rate [photons · s ⁻¹]						
	set 1	set 2	set 3	set 4	set 5	set 6	mean value [kcounts]
0	717	667	-	592	726	313	0.6 ± 0.2
0.02	-	1092	961	848	747	646	0.9 ± 0.2
0.04	1314	1224	1021	1045	1185	1035	1.1 ± 0.1
0.06	-	1683	1553	1743	1603	1463	1.6 ± 0.1
0.08	1920	2102	-	2084	1900	1688	1.9 ± 0.2
0.10	2113	2313	1837	-	-	1959	2.1 ± 0.2
0.12	-	2521	2416	2388	2136	2497	2.4 ± 0.1
0.14	2347	2859	2554	2940	2335	2702	2.6 ± 0.2
0.16	3120	3288	2779	3020	2504	-	2.9 ± 0.3
0.18	-	3364	3195	3233	2903	3281	3.2 ± 0.2
0.20	3551	3564	3403	3497	-	-	3.5 ± 0.1
0.40	6875	7081	6233	6595	6083	6360	6.5 ± 0.4
0.60	9731	9234	9167	9522	8196	9142	9.2 ± 0.5
0.80	12706	11642	11057	11885	10376	11493	11.5 ± 0.7
1	15794	14666	13681	13351	12399	12718	13.8 ± 1.2
1.5	20610	20021	18607	18672	16118	18535	18.8 ± 1.4
2	24831	24192	21553	22973	20542	22856	22.3 ± 1.5
2.5	28072	25490	24473	25847	21855	26673	25.4 ± 1.9
3	32856	30358	27713	27924	26197	-	29.0 ± 2.3
3.5	-	32910	30106	30193	28916	28704	30.2 ± 1.5
4	34665	33435	31339	32545	30599	31090	32.3 ± 1.4
4.5	-	36234	33572	33091	31737	32228	33.4 ± 1.6
5	37569	38914	34391	37037	33061	-	36.2 ± 2.2
10	45517	45166	47080	49200	38070	41447	44.4 ± 3.7
15	60294	46561	51569	55855	41783	47396	50.6 ± 6.2
20	63073	53203	56000	57748	43706	49832	53.9 ± 6.1
25	65041	53632	56614	60047	46432	52833	55.8 ± 5.9
30	67919	55149	59350	62970	47169	55760	58.1 ± 6.5
35	68070	55016	-	63546	47902	57290	58.4 ± 7.0
40	70251	55380	59873	64532	52514	58659	60.2 ± 5.8
45	71950	55307	61935	66289	54829	57552	61.3 ± 6.2
50	72657	63150	63478	66399	54766	58782	63.2 ± 5.6
500	-	70007	65807	76231	64627	68418	69.0 ± 4.1

Table 12: Collected photons per second for measured samples for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YO-PRO-1 dye was 40 nM. The dye concentration was kept constant. The concentration of DNA varies between 1 and 100 nM. " - "marks for the incorrectly prepared sample that did not result in the non-physical value of collected fluorescence intensity in the measurement.

DNA concentration [nM]	Count rate [photons · s ⁻¹]			
	set 1	set 2	set 3	mean value [kcounts]
0	1175	1078	1145	1.1 ± 0.1
1	1203	1075	-	1.1 ± 0.1
2	1382	1102	-	1.2 ± 0.1
3	1575	1403	2119	1.7 ± 0.3
4	1826	1679	2118	1.9 ± 0.2
5	1893	1849	-	1.9 ± 0.1
6	2347	2139	2367	2.3 ± 0.1
7	2592	-	2580	2.6 ± 0.1
8	-	2532	2981	2.8 ± 0.2
9	2733	2584	3704	3.0 ± 0.5
10	2996	3010	3856	3.3 ± 0.4
15	3974	3722	4179	4.0 ± 0.2
20	4876	4720	5483	5.0 ± 0.3
25	5662	5423	6438	5.8 ± 0.4
30	6338	6200	7373	6.6 ± 0.5
40	7592	7622	9542	8.3 ± 0.9
50	9162	8543	11066	9.6 ± 1.1
60	10526	-	13066	11.8 ± 1.3
70	12334	-	14603	13.5 ± 1.1
80	12682	-	15262	14.0 ± 1.3
90	14267	-	14697	14.5 ± 0.2
100	13902	-	15977	14.9 ± 1.0
500	28504	26799	32049	29.1 ± 2.2

Moreover, YOYO-1 consistently produces stronger fluorescence than YO-PRO-1 at equivalent DNA concentrations, indicating that YOYO-1 either possesses a higher binding affinity for DNA or undergoes a more pronounced fluorescence enhancement upon intercalation. This trend is evident at both investigated temperatures, suggesting that YOYO-1's superior performance is an intrinsic property of its interaction with DNA rather than a temperature-dependent effect.

Table 13: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YOYO-1 dye was 20 nM. The dye concentration was kept constant. The DNA concentration varies between 1 and 50 nM. "-" mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Count rate [photons · s ⁻¹]				
	set 1	set 2	set 3	set 4	mean value [kcounts]
0	-	790	595	535	0.6 ± 0.1
1	6220	7304	7338	5570	6.6 ± 0.7
1.5	8030	7891	7925	6240	7.5 ± 0.7
2	10121	10348	10382	-	10.3 ± 0.1
2.5	11760	12109	12143	8776	11.2 ± 1.4
3	13205	-	-	10214	11.7 ± 1.5
3.5	14842	14634	14668	11773	14.0 ± 1.3
4	16013	15786	15820	14665	15.6 ± 0.5
5	19538	17924	17958	16498	18.0 ± 1.1
10	28112	25784	25818	23938	25.9 ± 1.5
15	29922	26209	26243	27748	27.5 ± 1.5
20	-	29110	29144	-	29.1 ± 0.1
25	33881	30141	30175	-	31.4 ± 1.8
30	36285	-	-	29527	32.9 ± 3.4
40	36560	31140	31174	31281	32.5 ± 2.3
50	37757	31501	31535	32275	33.3 ± 2.6
500	42111	34715	32518	29975	34.8 ± 4.5

Table 14: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YO-PRO-1 dye was 40 nM. The dye concentration was kept constant. The concentration of tRNA varies between 1 and 200 nM. " - " mark for the incorrectly prepared sample that results in a non-physical value in the measurement.

tRNA concentration [nM]	Countrate [photons · s ⁻¹]			
	set 1	set 2	set 3	mean value [kcounts]
0	-	788	401	0.6 ± 0.2
1	720	-	681	0.7 ± 0.1
2	800	-	1082	0.9 ± 0.1
3	1252	933	-	1.1 ± 0.2
4	1206	1148	-	1.2 ± 0.1
5	-	1207	1291	1.2 ± 0.1
6	1476	1308	-	1.4 ± 0.1
7	-	1610	1366	1.5 ± 0.1
8	1836	-	1528	1.7 ± 0.2
9	-	1852	1610	1.7 ± 0.1
10	1846	1806	1833	1.8 ± 0.1
20	2741	2421	2857	2.7 ± 0.2
30	3730	3486	4060	3.8 ± 0.2
40	4289	4314	4747	4.5 ± 0.2
50	5891	5788	5272	5.7 ± 0.3
60	6702	6063	6499	6.4 ± 0.3
70	-	6956	6962	7.0 ± 0.1
80	-	7989	6969	7.5 ± 0.5
90	-	8196	9795	9.0 ± 0.8
100	-	9205	10837	10.0 ± 0.8
110	-	10705	10795	10.8 ± 0.1
120	-	11207	11306	11.3 ± 0.1
130	-	11761	13144	12.5 ± 0.7
140	-	13212	14055	13.6 ± 0.4
150	-	13825	15786	14.8 ± 1.0
160	-	15661	18066	16.9 ± 1.2
170	-	15833	18891	17.4 ± 1.5
180	-	17069	19832	18.5 ± 1.4
190	-	17934	20767	19.4 ± 1.4
200	-	19035	22633	20.8 ± 1.8
500	-	56272	42811	49.5 ± 6.7

To confirm the hypothesis that YO-PRO-1 dye follows a single, mono-intercalation mechanism, and that YOYO-1 dye intercalates in two separate modes of interactions, I closely monitored the changes in molecular brightness (MB), a key indicator of the extent of complex formation, as the DNA concentration increased across all sets of acquired data. Based on the theoretical number of dye molecules present in the focal volume, I calculated the molecular brightnesses of the studied reactions (Tables 15 - 19).

Molecular brightness is a quantitative measure of the fluorescence emitted per individual dye molecule and directly indicates the efficiency of complex formation. In this context, higher molecular brightness reflects more effective interactions

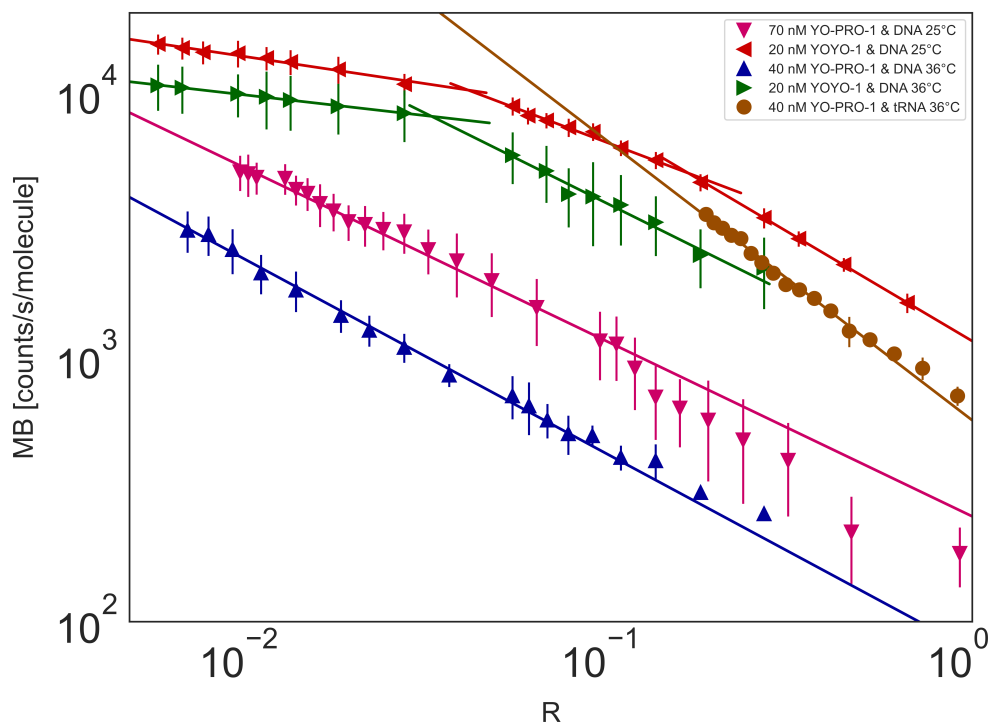


Fig. 24: The figure presents a log–log plot of molecular brightness (MB, in counts/s per molecule) versus the parameter R (ratio of dye to nucleic acids concentrations). The data points, shown with error bars, correspond to different dye–nucleic acid complexes and experimental conditions. The pink points ($\blacktriangledown$) correspond to 70 nM YO-PRO-1 bound to DNA at 25°C, the red points ($\blacktriangleleft$) to 20 nM YOYO-1 bound to DNA at 25°C, the blue points ($\blacktriangleup$) to 40 nM YO-PRO-1 bound to DNA at 36°C, the green ($\blacktriangleright$) points to 20 nM YOYO-1 bound to DNA at 36°C, and the brown points ($\bullet$) to 40 nM YO-PRO-1 bound to tRNA at 36°C. Straight lines are fitted to each dataset to capture the overall scaling behaviour. The y-axis spans from 10^2 to 10^4 counts/s per molecule, while the x-axis covers R values from approximately 10^{-2} to 1.

between the dye and its nucleic acid target, whether in DNA–dye complexes or in the case of YO-PRO-1, its binding to tRNA.

The log–log representation reveals pronounced differences in molecular brightness among the studied dyes and binding conditions. Overall, molecular brightness decreases systematically with increasing R , the ratio of cyanine dye to nucleic acid concentration (Fig. 24), consistent with a power-law relationship reflecting reduced fluorescence efficiency at higher binding ratios.

Based on Fig. 24, the fluorescence response of YO-PRO-1 indicates that its interaction with nucleic acids follows a single, mono-intercalation mechanism. Furthermore, increasing the temperature leads to a noticeable reduction in the molecular brightness of the YO-PRO-1–DNA complex, suggesting that elevated thermal energy weakens either the stability of the intercalated state or the bound dye’s fluorescence efficiency.

However, the reaction of YO-PRO-1 with tRNA is characterised by the highest molecular brightness among all investigated YO-PRO-1–nucleic acid complexes. The robust fluorescence of the tRNA–YO-PRO-1 complex may be attributed to the specific structural arrangement of the dye upon binding to tRNA, which could reduce non-radiative decay pathways. In particular, restricted or altered rotational motion of the intercalated dye within the compact tRNA structure is likely to enhance the quantum yield, producing a markedly brighter signal than that of DNA-bound complexes.

YOYO-1, a homodimer of YO-PRO-1, contains two fluorescent moieties capable of intercalating independently into nucleic acids. This structural feature allows the distinction between different binding regimes. At both 25°C and 36°C, a mono-intercalation regime is observed at higher binding ratios ($R > 0.20$). A bis-intercalation regime dominates at lower binding ratios ($R < 0.045$). Between these thresholds lies an intermediate region, where both mono- and bis-intercalated complexes coexist (Fig. 24).

Table 15: Calculated molecular brightness (MB) (Eq. 4) for measured samples of DNA-YO-PRO-1 formed complex, for all sets of titration experiments, performed at 25°C, in equilibrium state. The initial concentration of YO-PRO-1 dye was 70 nM and was kept constant. The concentration of the DNA (69 bp) was varied from 0.5 to 200 nM. " - "marks the incorrectly prepared sample, resulting in a non-physical value of the collected fluorescence intensity.

DNA concentration [nM]	Molecular Brightness [$\frac{\text{counts}}{\text{molecule} \cdot \text{s}}$]						mean value [$\frac{\text{kcounts}}{\text{molecule} \cdot \text{s}}$]
	set 1	set 2	set 3	set 4	set 5	set 6	
0.5	133	132	-	-	-	276	0.2 ± 0.1
1	177	141	235	121	232	-	0.2 ± 0.1
2	-	191	-	138	-	326	0.2 ± 0.1
3	299	657	-	195	466	417	0.4 ± 0.2
4	370	864	324	242	607	510	0.5 ± 0.2
5	381	1025	395	348	697	616	0.6 ± 0.2
6	470	-	567	446	854	869	0.6 ± 0.2
7	560	-	651	535	-	1077	0.7 ± 0.2
8	616	1328	749	581	1049	1126	0.9 ± 0.3
9	-	1410	-	614	1151	1302	1.1 ± 0.3
10	900	1508	-	746	-	1450	1.2 ± 0.3
15	1212	1997	1197	929	1968	1934	1.5 ± 0.4
20	1655	2384	1453	1204	2522	2445	1.9 ± 0.5
25	1964	2776	1809	1374	3090	2841	2.3 ± 0.6
30	2508	-	2038	1978	3135	3071	2.6 ± 0.5
35	-	-	-	2245	3316	3355	3.0 ± 0.5
40	3026	2934	2462	2451	3741	3615	3.0 ± 0.5
45	3404	3136	-	2533	-	3920	3.3 ± 0.5
50	-	3296	-	2755	4218	3991	3.6 ± 0.6
55	4075	-	3218	2785	4709	4218	3.8 ± 0.7
60	-	3698	3527	-	4997	4410	4.2 ± 0.6
65	-	3851	3681	-	5030	4634	4.3 ± 0.6
70	-	3886	-	-	5377	4945	4.7 ± 0.6
75	-	3990	4017	3529	5589	5107	4.5 ± 0.8
80	4263	4052	4211	3747	5633	5283	4.5 ± 0.7
85	4323	4113	4289	-	5700	5443	4.8 ± 0.7
90	-	4414	-	3763	6056	5496	4.9 ± 0.9
95	-	4567	4451	4193	6115	5717	5.0 ± 0.8
100	-	4646	4611	4339	6134	5903	5.1 ± 0.7
120	4361	4867	4682	4394	6723	6270	5.2 ± 0.9
140	-	5093	-	-	6792	6547	6.1 ± 0.8
160	5675	5392	5163	5227	7324	6667	5.9 ± 0.8
180	5940	5564	5422	-	7627	7092	6.3 ± 0.9
200	6225	-	5593	5597	7317	7512	6.5 ± 0.8
500	7454	8080	8233	6872	10321	9427	8.4 ± 1.2

Table 16: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 25°C, in equilibrium state. The initial concentration of YOYO-1 dye was 20 nM. The dye concentration was kept constant. The DNA concentration ranges from 0.02 to 50 nM. "-" marks for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Molecular Brightness [$\frac{\text{counts}}{\text{molecule} \cdot \text{s}}$]						mean value [$\frac{\text{kcounts}}{\text{molecule} \cdot \text{s}}$]
	set 1	set 2	set 3	set 4	set 5	set 6	
0.02	111	275	220	222	188	153	0.2 ± 0.1
0.04	309	311	236	262	311	238	0.3 ± 0.1
0.06	418	433	343	452	400	335	0.4 ± 0.1
0.08	451	544	438	541	480	392	0.5 ± 0.1
0.10	496	589	-	-	585	452	0.5 ± 0.1
0.12	-	637	554	631	-	594	0.6 ± 0.1
0.14	551	721	586	754	606	644	0.6 ± 0.1
0.16	733	826	639	767	660	404	0.7 ± 0.1
0.18	-	847	729	823	741	764	0.8 ± 0.1
0.20	834	895	783	898	-	719	0.8 ± 0.1
0.40	1615	1840	1422	1686	1569	1483	1.6 ± 0.1
0.60	2286	2355	2093	2443	2117	2125	2.2 ± 0.1
0.80	2984	2933	2557	3035	2646	2665	2.8 ± 0.2
1	3710	3738	3153	3421	3176	2954	3.4 ± 0.3
1.5	4841	5076	4260	4782	4154	4326	4.6 ± 0.3
2	5833	6138	4942	5840	5264	5288	5.5 ± 0.4
2.5	6594	6440	5618	6569	5582	6223	6.2 ± 0.4
3	7718	7696	6343	7101	6684	-	7.1 ± 0.5
3.5	-	8315	6854	7675	7388	6682	7.4 ± 0.6
4	8143	8484	7148	8328	7781	7199	7.8 ± 0.5
4.5	-	9171	7659	8431	8134	7492	8.2 ± 0.6
5	8825	9816	7867	9471	8443	-	8.9 ± 0.7
10	10692	11364	10712	12527	9702	9540	10.8 ± 1.0
15	14163	11696	11723	14190	10654	10949	12.2 ± 1.4
20	14816	13402	12739	14694	11130	11546	13.1 ± 1.4
25	15278	13505	12901	15279	11814	12194	13.5 ± 1.4
30	15954	-	13500	15994	12045	12864	14.1 ± 1.6
35	15990	13839	13721	16137	12240	13254	14.2 ± 1.4
40	16505	13924	-	16358	13334	13541	14.7 ± 1.4
45	16901	13944	14099	16879	13964	-	15.2 ± 1.4
50	17067	15880	14443	-	-	13593	15.3 ± 1.3

Table 17: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium constant. The initial concentration of YO-PRO-1 dye was 40 nM, and was kept constant. The concentration of DNA varies between 1 and 100 nM. " - "marks for the incorrectly prepared sample that did not result in the proper value of collected fluorescence intensity in the measurement.

DNA concentration [nM]	Molecular Brightness [$\frac{\text{counts}}{\text{molecule} \cdot \text{s}}$]			
	set 1	set 2	set 3	mean value [$\frac{\text{kcunts}}{\text{molecule} \cdot \text{s}}$]
1	235	236	-	0.2 ± 0.1
2	270	242	-	0.3 ± 0.1
3	308	308	-	0.3 ± 0.1
4	357	368	493	0.4 ± 0.1
5	370	406	479	0.4 ± 0.1
6	459	-	551	0.5 ± 0.1
7	-	427	601	0.5 ± 0.1
8	487	555	694	0.6 ± 0.1
9	534	567	863	0.7 ± 0.1
10	586	660	898	0.7 ± 0.1
15	777	817	973	0.9 ± 0.1
20	954	1035	1277	1.1 ± 0.1
25	1107	1189	1499	1.3 ± 0.2
30	1239	1360	1717	1.4 ± 0.2
40	1485	1672	2222	1.8 ± 0.3
50	1792	1874	2577	2.1 ± 0.4
60	2059	-	3043	2.6 ± 0.5
70	2412	-	3401	2.9 ± 0.5
80	2480	-	3555	3.0 ± 0.5
90	2790	-	3423	3.1 ± 0.3
100	2719	-	3721	3.2 ± 0.5
500	5574	5879	7465	6.3 ± 0.8

Again, apparent differences can be observed with increasing temperature. Similar to the YO-PRO-1 dye, elevated temperature decreases the molecular brightness of the YOYO-1–DNA complex, indicating reduced fluorescence efficiency under thermal stress. In addition, at higher temperatures, the balance between binding modes shifts, with bis-intercalation becoming the dominant reaction pathway already at comparatively higher DNA concentrations. This suggests that increased thermal energy facilitates conformational adjustments of the complex, promoting the dual intercalation of both fluorophores in YOYO-1.

Table 18: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YOYO-1 dye was 20 nM. The dye concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - " mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Molecular Brightness [$\frac{\text{counts}}{\text{molecule} \cdot \text{s}}$]				
	set 1	set 2	set 3	set 4	mean value [$\frac{\text{kcounts}}{\text{molecule} \cdot \text{s}}$]
0	265	285	231	197	0.2 ± 0.1
1	1158	2638	2848	2056	2.2 ± 0.7
1.5	1495	2850	3076	2303	2.4 ± 0.6
2	1885	3737	4029	3283	3.2 ± 0.8
2.5	2190	4373	4713	-	3.8 ± 1.1
3	2459	-	5895	3770	4.0 ± 1.4
3.5	2764	5284	-	4346	4.1 ± 1.0
4	2982	5700	6140	5414	5.1 ± 1.2
5	3639	6473	6970	6090	5.8 ± 1.3
10	5235	9311	10020	8836	8.4 ± 1.8
15	5572	9464	10185	10243	8.9 ± 1.9
20	6299	10512	11311	-	9.4 ± 2.2
25	6309	10844	11711	-	9.6 ± 2.4
30	6757	10551	11353	10899	9.9 ± 1.8
40	6808	11245	12099	11547	10.4 ± 2.1
50	7031	11376	12239	11914	10.6 ± 2.1

Table 19: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of YO-PRO-1 dye was 40 nM. The dye concentration was kept constant. The concentration of tRNA varies between 1 and 200 nM. " - " mark for the incorrectly prepared sample that results in a non-physical value in the measurement.

tRNA concentration [nM]	Molecular Brightness [$\frac{\text{counts}}{\text{molecule} \cdot \text{s}}$]			
	set 1	set 2	set 3	mean value [$\frac{\text{kcounts}}{\text{molecule} \cdot \text{s}}$]
1	106	-	106	0.1 ± 0.1
2	118	-	169	0.1 ± 0.1
3	185	166	-	0.2 ± 0.1
4	178	204	-	0.2 ± 0.1
5	-	215	202	0.2 ± 0.1
6	218	233	-	0.2 ± 0.1
7	-	286	214	0.3 ± 0.1
8	271	-	239	0.3 ± 0.1
9	-	330	252	0.3 ± 0.1
10	272	321	287	0.3 ± 0.1
20	404	431	447	0.4 ± 0.1
30	550	620	635	0.6 ± 0.1
40	633	768	743	0.7 ± 0.1
50	869	1030	825	0.9 ± 0.1
60	989	1079	1017	1.0 ± 0.1
70	-	1238	1090	1.2 ± 0.1
80	-	1421	1091	1.3 ± 0.2
90	-	1458	1533	1.5 ± 0.1
100	-	1638	1696	1.7 ± 0.1
110	-	1905	1689	1.8 ± 0.1
120	-	1994	1769	1.9 ± 0.1
130	-	2093	2057	2.1 ± 0.1
140	-	2351	2199	2.3 ± 0.1
150	-	2460	2470	2.5 ± 0.1
160	-	2787	2827	2.8 ± 0.1
170	-	2817	2956	2.9 ± 0.1
180	-	3037	3104	3.1 ± 0.1
190	-	3191	3250	3.2 ± 0.1
200	-	3387	3542	3.5 ± 0.1
500	-	10013	6700	8.4 ± 1.7

Anthracyclines: doxorubicin, daunorubicin, idarubicin and epirubicin

In this section, I described the reactions between DNA (69 bp) and the anthracycline drugs doxorubicin (DOX), daunorubicin (DAU), idarubicin (IDA), and epirubicin (EPI). I compared the results obtained at 25°C by Zhou and colleagues [188] to investigate the influence of temperature on the complex-formation reaction.

I performed titration experiments at 36°C. The drug concentration was kept constant (100 nM), and DNA concentration varied between 1 and 50 nM for all four anthracyclines studied. The plot in Fig. 25 shows the change in fluorescence intensity upon increasing DNA concentration.

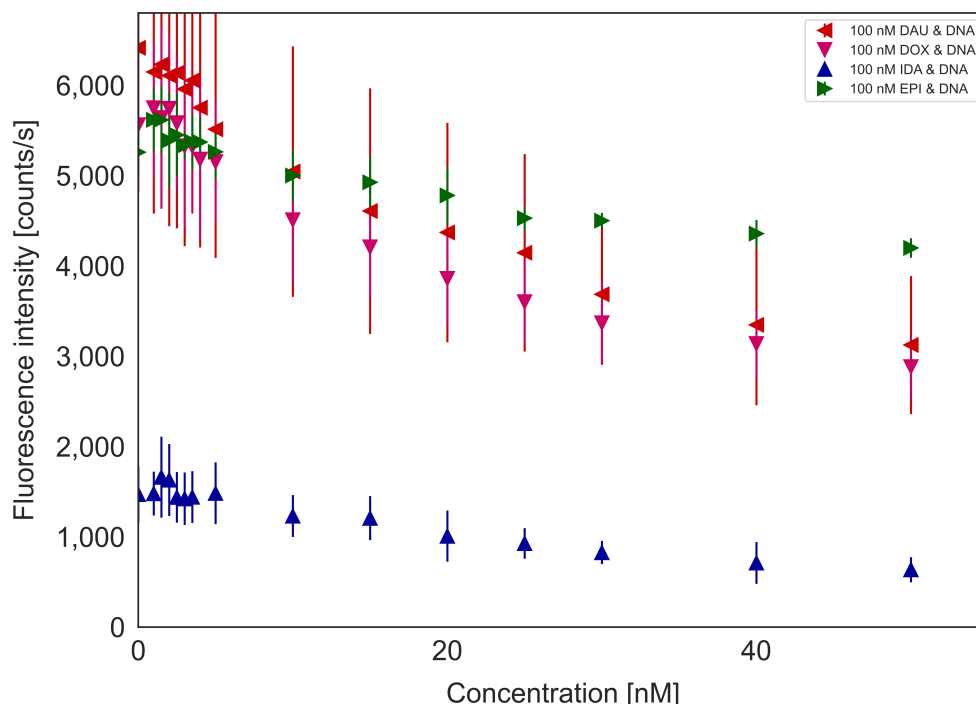


Fig. 25: Fluorescence titration of anthracyclines with DNA (69 bp, 36°C). Fluorescence intensity (counts/s) of 100 nM solutions of four anthracycline drugs – daunorubicin (DAU, red ◄), doxorubicin (DOX, pink ▼), idarubicin (IDA, blue ▲), and epirubicin (EPI, green ►) – is plotted against increasing DNA concentration (nM). All four dyes exhibit a decrease in fluorescence intensity as more DNA is added, consistent with fluorescence quenching upon drug–DNA binding. Epirubicin shows the highest fluorescence across the titration range, whereas idarubicin gives the lowest signal, with doxorubicin and daunorubicin intermediate. Error bars denote experimental variability.

Recorded fluorescence intensities from all consecutive experiments and samples are presented in Tables 20 - 23 (DOX - Table 20, DAU - Table 21, IDA - Table 22, EPI - Table 23).

All four anthracyclines demonstrate a monotonic decrease in fluorescence intensity with increasing DNA concentration. At zero DNA concentration, the dyes exhibit maximal or near-maximal fluorescence in free solution. As DNA is added, fluorescence steadily drops for each compound, eventually approaching a lower plateau at high DNA concentrations. Binding to DNA quenches the drug’s fluorescence rather than enhancing it – a phenomenon reported for anthracyclines like doxorubicin (in contrast to classical intercalators such as ethidium bromide, which become more fluorescent upon intercalation).

Among the anthracyclines investigated, epirubicin displays the highest fluorescence intensity and is therefore the least quenched when bound to DNA. Its emission remains relatively strong even as DNA concentration increases, suggesting that its binding orientation or local environment within the DNA helix allows it to retain a larger fraction of its intrinsic fluorescence.

In contrast, doxorubicin and daunorubicin show similar fluorescence behaviours, starting at moderate intensities and exhibiting comparable quenching trends with increasing DNA concentration. Their nearly overlapping responses indicate that, despite minor structural differences, the two drugs interact with DNA in a similar manner.

Idarubicin, however, is the weakest emitter by a considerable margin. Its fluorescence is markedly lower across all DNA concentrations, reflecting much stronger

Table 20: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of doxorubicin was 100 nM. The drug concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - " mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Count rate [photons · s ⁻¹]				
	set 1	set 2	set 3	set 4	mean value [kcounts]
0	5928	4836	4881	6597	5.6 ± 0.7
1	5689	-	4862	6691	5.7 ± 0.7
1.5	-	4856	5001	7056	5.6 ± 1.0
2	6429	4840	5030	6670	5.7 ± 0.8
2.5	5873	4898	4893	6659	5.6 ± 0.7
3	-	4602	4770	6606	5.3 ± 0.9
3.5	5400	4551	4844	6500	5.3 ± 0.7
4	-	4440	4654	6439	5.2 ± 0.9
5	5337	4488	4629	6148	5.2 ± 0.7
10	4429	3887	4044	5668	4.5 ± 0.7
15	4300	3682	3756	5094	4.2 ± 0.6
20	3866	3421	3493	4650	3.9 ± 0.5
25	3531	3191	3308	4374	3.6 ± 0.5
30	3192	3051	3059	4160	3.4 ± 0.5
40	2955	2824	2997	3771	3.1 ± 0.4
50	2737	2594	2713	3477	2.9 ± 0.3
500	1860	-	1827	2275	2.0 ± 0.2

quenching or inherently lower quantum yield in the DNA-bound state.

The fluorescence quenching behaviour observed here, decreasing intensity as DNA concentration increases, is consistent with previous studies performed at 25°C [188]. This agreement suggests that the fundamental photophysical interactions between anthracyclines and DNA — dominated by intercalation and subsequent quenching — are largely preserved across this temperature range, with only minor variations in quenching extent among different drugs.

Table 21: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial daunorubicin concentration was 100 nM. The drug concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Count rate [photons · s ⁻¹]					
	set 1	set 2	set 3	set 4	set 5	mean value [kcounts]
0	-	-	4986	8168	6089	6.4 ± 1.3
1	5213	4464	-	8619	6293	6.1 ± 1.6
1.5	4921	-	4817	8645	6532	6.2 ± 1.5
2	-	4388	4820	8604	6612	6.1 ± 1.7
2.5	4986	4234	-	8720	6608	6.1 ± 1.7
3	4811	-	4646	8415	-	6.0 ± 1.7
3.5	4863	-	4678	8111	6559	6.1 ± 1.4
4	-	4232	4570	8156	6045	5.8 ± 1.5
5	4639	4295	4589	8143	5892	5.5 ± 1.4
10	4034	4082	4165	7660	5284	5.0 ± 1.4
15	3971	3737	3762	6957	-	4.6 ± 1.4
20	3740	3482	3377	6638	4609	4.4 ± 1.2
25	3359	3096	-	5892	4234	4.1 ± 1.1
30	3210	2989	3091	5027	4102	3.7 ± 0.8
40	2733	2549	2743	4888	3822	3.3 ± 0.9
50	2545	2557	2535	4440	3544	3.1 ± 0.8
500	929	749	779	686	1046	0.8 ± 0.1

Table 22: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of idarubicin was 100 nM. The drug concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Count rate [photons · s ⁻¹]			
	set 1	set 2	set 3	mean value [kcnts]
0	1908	1301	1201	1.5 ± 0.3
1	1820	1280	1336	1.5 ± 0.2
1.5	2293	1397	1295	1.7 ± 0.4
2	2175	1479	1234	1.6 ± 0.4
2.5	1832	1286	1196	1.4 ± 0.3
3	1830	1256	1181	1.4 ± 0.3
3.5	1843	1291	1190	1.4 ± 0.3
5	1824	-	1140	1.5 ± 0.3
10	1554	1116	1022	1.2 ± 0.2
15	1451	-	963	1.2 ± 0.2
20	1395	727	901	1.0 ± 0.3
25	1097	-	758	0.9 ± 0.2
30	1006	753	719	0.8 ± 0.1
40	942	-	480	0.7 ± 0.2
50	812	625	471	0.6 ± 0.1
500	266	195	217	0.2 ± 0.1

Table 23: Measured samples and collected photons per second upon complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial epirubicin concentration was 100 nM. The drug concentration was kept constant. The DNA concentration varies between 1 and 50 nM. " - " mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Countrate [photons · s ⁻¹]			
	set 1	set 2	set 3	mean value [kcnts]
0	5502	5012	-	5.3 ± 0.2
1	6094	5460	5296	5.6 ± 0.3
1.5	6031	5684	5137	5.6 ± 0.4
2	5920	-	4856	5.4 ± 0.5
2.5	5904	-	4994	5.4 ± 0.5
3	-	5478	5187	5.3 ± 0.1
3.5	5777	5304	5038	5.4 ± 0.3
4	5760	5265	5087	5.4 ± 0.3
5	5625	5253	4908	5.3 ± 0.3
10	5399	4853	4748	5.0 ± 0.3
15	5309	4812	4655	4.9 ± 0.3
20	5165	4780	4395	4.8 ± 0.3
25	4680	4542	4359	4.5 ± 0.1
30	4586	4412	-	4.5 ± 0.1
40	4508	-	4206	4.4 ± 0.2
50	4199	4330	4066	4.2 ± 0.1
500	3420	3443	3215	3.4 ± 0.1

Next, I investigated the mechanisms of anthracycline-DNA reactions at 36°C. I collected calculated molecular brightnesses for all consecutive samples and sets of data in Tables 24 - 27 (DOX - Table 24, DAU - Table 25, IDA - Table 26, EPI - Table 27).

The plot (Fig. 26) highlights distinct differences in molecular brightness among the four anthracyclines when bound to DNA. Epirubicin (red) shows the highest molecular brightness across the full R range, maintaining values of $3\text{-}5 \times 10^2$ counts/s per molecule. Daunorubicin (pink) and doxorubicin (cyan) display nearly overlapping trends, with MB values only slightly lower than those of epirubicin, suggesting that both drugs exhibit similar fluorescence efficiencies in their DNA-bound states.

By contrast, idarubicin (blue) consistently shows the lowest molecular brightness, an order of magnitude weaker than the other three anthracyclines (approximately 3×10^1 to 1×10^2 counts/s per molecule). This confirms that idarubicin is strongly quenched upon DNA binding, or inherently less fluorescent in the DNA-bound state.

The relatively flat slopes of the curves for epirubicin, daunorubicin, and doxorubicin suggest that their photophysical properties remain essentially constant across different binding ratios. Idarubicin shows a modest upward trend with increasing R, but the absolute brightness remains much lower than the others.

The molecular brightness data match the fluorescence intensity results, showing that epirubicin forms the brightest complexes, doxorubicin and daunorubicin behave similarly, and idarubicin remains the least emissive due to strong quenching effects.

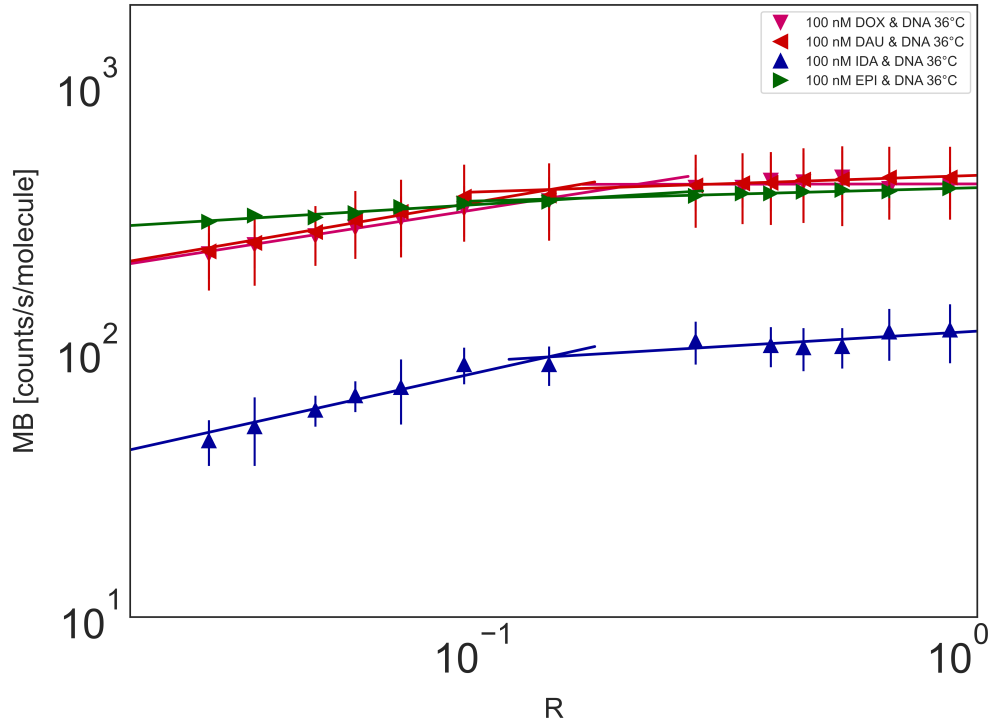


Fig. 26: The figure presents the molecular brightness (MB, counts/s per molecule) of four anthracyclines — daunorubicin (DAU, red ◄), doxorubicin (DOX, pink ▼), idarubicin (IDA, blue ►), and epirubicin (EPI, green ▲) — as a function of the binding ratio R with 69 bp DNA at 36°C. Both axes are plotted on logarithmic scales. Data points are shown with error bars, and fitted lines illustrate the overall trends. The y-axis spans from 10^1 to 10^3 counts/s per molecule, while the x-axis ranges from approximately 10^{-2} to 1.

Table 24: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of doxorubicin was 100 nM. The drug concentration was kept constant. The concentration of DNA varies between 1 and 500 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Molecular Brightness [$\frac{\text{counts}}{\text{molecule} \cdot \text{s}}$]					
	set 1	set 2	set 3	set 4	set 5	mean value [cnts $\times 10^2$]
0	437	-	365	-	488	4.3 ± 0.5
1	419	343	363	638	-	4.4 ± 1.2
1.5	-	355	374	-	522	4.2 ± 0.8
2	-	354	376	-	494	4.1 ± 0.6
2.5	433	358	365	645	-	4.5 ± 1.2
3	-	336	356	605	-	4.3 ± 1.2
3.5	398	332	362	615	481	4.4 ± 1.0
4	-	-	348	-	477	4.1 ± 0.7
5	393	328	346	537	455	4.1 ± 0.8
10	326	284	302	504	420	3.7 ± 0.8
15	317	269	280	470	377	3.4 ± 0.7
20	285	250	261	430	344	3.1 ± 0.7
25	260	233	247	390	324	2.9 ± 0.6
30	235	223	228	363	308	2.7 ± 0.6
40	218	206	224	328	279	2.5 ± 0.5
50	202	190	203	308	257	2.3 ± 0.5
500	137	-	136	154	168	1.5 ± 0.1

Table 25: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial daunorubicin concentration was 100 nM. The drug concentration was kept constant. The concentration of DNA varies between 1 and 500 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Molecular Brightness [$\frac{\text{counts}}{\text{molecule} \cdot \text{s}}$]					
	set 1	set 2	set 3	set 4	set 5	mean value [cnts $\times 10^2$]
0	352	310	372	664	451	4.3 ± 1.3
1	384	326	360	701	466	4.5 ± 1.4
1.5	363	334	360	703	483	4.5 ± 1.4
2	377	321	360	700	489	4.5 ± 1.4
2.5	367	309	335	709	489	4.4 ± 1.5
3	355	316	347	684	505	4.4 ± 1.4
3.5	358	293	349	659	485	4.3 ± 1.3
4	372	309	341	663	447	4.3 ± 1.3
5	342	314	343	662	436	4.2 ± 1.3
10	297	298	311	623	391	3.8 ± 1.2
15	293	273	281	566	487	3.8 ± 1.2
20	276	254	252	540	341	3.3 ± 1.1
25	248	226	291	479	313	3.1 ± 0.9
30	237	218	231	409	304	2.8 ± 0.7
40	201	186	205	397	283	2.6 ± 0.8
50	188	187	189	361	262	2.4 ± 0.7
500	68	55	58	56	77	0.6 ± 0.1

Table 26: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial concentration of idarubicin was 100 nM. The drug concentration was kept constant. The concentration of DNA varies between 1 and 500 nM. " - "mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Molecular Brightness [$\frac{\text{counts}}{\text{molecule} \cdot \text{s}}$]			
	set 1	set 2	set 3	mean value [cnts $\times 10^2$]
0	135	92	93	1.1 ± 0.2
1	129	90	104	1.1 ± 0.2
1.5	162	99	100	1.2 ± 0.3
2	154	104	96	1.2 ± 0.3
2.5	130	91	93	1.0 ± 0.2
3	130	89	92	1.0 ± 0.2
3.5	131	91	92	1.1 ± 0.2
5	129	-	88	1.1 ± 0.2
10	110	79	79	0.9 ± 0.2
15	103	-	75	0.9 ± 0.1
20	99	51	70	0.7 ± 0.2
25	78	-	59	0.7 ± 0.1
30	71	53	56	0.6 ± 0.1
40	67	-	37	0.5 ± 0.2
50	58	44	37	0.5 ± 0.1
500	19	14	17	0.2 ± 0.1

Table 27: Calculated molecular brightness (MB) for measured samples after the complex formation for all sets of titration experiments, performed at 36°C, in equilibrium state. The initial epirubicin concentration was 100 nM. The drug concentration was kept constant. The concentration of DNA varies between 1 and 500 nM. "-" mark for the incorrectly prepared sample that did not result in a non-physical value in the measurement.

DNA concentration [nM]	Molecular Brightness [$\frac{\text{counts}}{\text{molecule} \cdot \text{s}}$]			
	set 1	set 2	set 3	mean value [cnts $\times 10^2$]
0	390	354	-	3.7 ± 0.2
1	432	385	411	4.1 ± 0.2
1.5	427	401	398	4.1 ± 0.1
2	420	-	376	4.0 ± 0.2
2.5	418	-	387	4.0 ± 0.2
3	402	387	402	4.0 ± 0.1
3.5	409	374	391	3.9 ± 0.1
4	408	372	394	3.9 ± 0.2
5	399	371	380	3.8 ± 0.1
10	383	342	368	3.6 ± 0.2
15	376	340	361	3.6 ± 0.2
20	366	337	341	3.5 ± 0.1
25	332	321	338	3.3 ± 0.1
30	325	311	-	3.2 ± 0.1
40	319	-	326	3.2 ± 0.1
50	298	306	315	3.1 ± 0.1
500	242	243	249	2.5 ± 0.1

3.1.3 Equilibrium constant determination

Despite the mechanisms of the intercalation reactions being studied, the ultimate goal was to characterise the reactions quantitatively by determining equilibrium constants.

Cyanine dyes: YO-PRO-1 and YOYO-1

First, I calculated the molecular brightnesses of the free dyes and the formed complexes for each experiment. Then, I applied the Brightness Analysis Method (BAM) to obtain equilibrium constants for the studied reactions using molecular brightnesses from consecutive experiments.

In Table 28, I collected the molecular brightnesses and equilibrium constants obtained from YO-PRO-1 reactions with DNA and tRNA, at both 25°C and 36°C, from all consecutive measurements. And in Table 29, I collected the molecular brightnesses and equilibrium constants obtained from YOYO-1 reactions with DNA, at both 25°C and 36°C, from all consecutive measurements.

The molecular brightness of the free YO-PRO-1 and YOYO-1 dyes, α , comes from the scattering of the laser on the dye's molecules. Notably, at 36°C, an apparent increase in the molecular brightness of the free dyes was observed compared to 25°C. This increase is likely due to enhanced laser-scattering effects, which are temperature-dependent and may arise from changes in the molecular motion or conformation of the dyes at higher temperatures. This observation suggests that temperature influences the scattering properties of free dyes, potentially affecting the interpretation of fluorescence data during complex formation.

In contrast, the increase in temperature led to a decrease in the molecular brightness of the YO-PRO-1 complex bound to DNA (γ); thus, the decrease falls within the margin of error.

YOYO-1 dye interacts in two modes, mono- and bis-intercalation. I calculated two molecular brightnesses of the complexes and characterised their formation reactions with distinct equilibrium constants.

As expected, the bis-intercalated complex has a higher molecular brightness, doubling that of the mono-intercalated complex.

As observed in Tables 28 and 29, at 25°C, the molecular brightness of the mono-intercalated complex of YOYO-1 and of YO-PRO-1 is pretty similar, indicating comparable binding efficiencies for both dyes in their interaction with DNA. However, when the temperature was increased to 36°C, this pattern changed. YO-PRO-1 exhibited a higher molecular brightness in its DNA complex at higher temperatures than YOYO-1.

This shift in molecular brightness could be attributed to structural differences between the two dyes. Specifically, the second "mer" in YOYO-1 may affect the complex's electron cloud as temperature increases, potentially altering intercalation dynamics. The second aromatic group in YOYO-1 might influence electron density more pronouncedly at elevated temperatures, leading to changes in fluorescence characteristics.

Interestingly, both dyes showed decreased molecular brightness for their complexes with DNA at higher temperatures. It is consistent with the literature [152] and may be associated with greater rotational freedom of aromatic groups linked to the linker.

Table 28: Compiled equilibrium constants and molecular brightnesses obtained from the analysed experimental data of the reactions between DNA and YO-PRO-1, and tRNA and YO-PRO-1, carried out at 25°C and 36°C, respectively.

SET	25°C			36°C					
	DNA						tRNA		
	α [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ [$\frac{\text{kcnts}}{\text{mol.} \cdot \text{s}}$]	K [$\times 10^6$] [M^{-1}]	α [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ [$\frac{\text{kcnts}}{\text{mol.} \cdot \text{s}}$]	K [$\times 10^5$] [M^{-1}]	α [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ [$\frac{\text{kcnts}}{\text{mol.} \cdot \text{s}}$]	K [$\times 10^5$] [M^{-1}]
1	47	7.5	0.9	114	5.6	4.0	53	14.9	6.9
2	59	8.1	2.0	61	5.9	3.9	59	15.1	7.8
3	53	8.2	1.0	121	7.5	7.3	48	18.0	7.8
4	42	6.9	0.7	-	-	-	-	16.9	-
5	61	10.3	1.1	-	-	-	-	-	-
6	61	9.4	0.1	-	-	-	-	-	-
AVG	54 $\pm$ 7	8.4 $\pm$ 1.2	1.0 $\pm$ 0.6	99 $\pm$ 27	6.3 $\pm$ 0.8	3.9 $\pm$ 0.1	53 $\pm$ 4	16.2 $\pm$ 1.3	7.5 $\pm$ 0.4

Comparing the molecular brightnesses obtained for YO-PRO-1 complexes with DNA and tRNA, the molecular brightness of the tRNA-YO-PRO-1 complex is almost 3 times bigger than that of the complex with DNA at the same temperature. The significant impact of tRNA’s rotational motion could explain it.

This significant difference in molecular brightness is likely due to the unique rotational dynamics of tRNA within the focal volume during the experiment. Unlike DNA, which remains relatively rigid, tRNA exhibits more rotational motion due to its three-dimensional structure. This increased mobility leads to more excitation and emission events, ultimately resulting in higher fluorescence. This difference highlights the influence of molecular mobility on the fluorescence signal and emphasises the importance of considering such factors when analysing molecular interactions.

To better determine the equilibrium constants, I refitted the data, using averaged molecular brightnesses. The results of the refitting are collected in the Table 30, while exemplary fitting plots are shown in Figures 27 - 33.

However, refitting did not change the equilibrium constant values much, keeping the new values within error.

I observed a general decrease of approximately one order of magnitude for all the complexes formed at 36°C, compared to the values measured at 25°C. Interestingly, the mono-intercalated YOYO-1 complex remained relatively stable, showing little change in its equilibrium constant with temperature variation. This suggests that the intercalation of YOYO-1 into DNA is less affected by temperature than the other complex formations.

YO-PRO-1 complexes can be characterised by an equilibrium constant value that falls between the mono- and bis-intercalated forms of YOYO-1. This intermediate value reflects the unique behaviour of YO-PRO-1 in complex formation, potentially due to its structural differences from YOYO-1 and to how these differences influence binding efficiency and temperature response.

Interestingly, despite the significant difference in molecular brightnesses of formed complexes, the equilibrium constants for YO-PRO-1 reactions with DNA and tRNA are in the same order.

The results underscore the significant impact of both the reaction mechanism

Table 29: Compiled equilibrium constants and molecular brightnesses obtained from the analysed experimental data of the reactions between DNA and YOYO-1, carried out at 25°C and 36°C, separately. Equilibrium constants for the reactions of DNA are calculated based on base-pair concentration.

SET	25°C				
	α	γ_1	γ_2	$K_1 [\times 10^7]$	$K_2 [\times 10^5]$
	$[\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}]$	$[\frac{\text{kcnts}}{\text{mol.} \cdot \text{s}}]$	$[\frac{\text{kcnts}}{\text{mol.} \cdot \text{s}}]$	$[\text{M}^{-1}]$	$[\text{M}^{-1}]$
1	53	10.6	21.3	2.5	1.9
2	38	8.8	17.6	3.5	1.1
3	30	7.5	15.0	3.7	2.1
4	36	9.7	19.3	3.6	2.4
5	14	8.2	16.4	3.0	1.3
6	18	7.9	15.8	4.0	2.2
AVG	32 ± 13	8.8 ± 1.1	17.6 ± 2.2	3.4 ± 0.5	1.9 ± 0.5
SET	36°C				
	α	γ_1	γ_2	$K_1 [\times 10^7]$	$K_2 [\times 10^4]$
	$[\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}]$	$[\frac{\text{kcnts}}{\text{mol.} \cdot \text{s}}]$	$[\frac{\text{kcnts}}{\text{mol.} \cdot \text{s}}]$	$[\text{M}^{-1}]$	$[\text{M}^{-1}]$
1	53	3.9	7.8	1.1	2.4
2	97	6.3	12.5	1.9	4.1
3	125	6.3	12.6	2.0	4.7
4	108	5.5	11.1	2.2	-
AVG	96 ± 27	5.5 ± 1.0	11.0 ± 1.9	1.8 ± 0.4	3.7 ± 1.0

Table 30: Compiled equilibrium constants, fitted with averaged molecular brightnesses, of the reactions between YO-PRO-1 with DNA or tRNA, and DNA with YOYO-1, carried out at 25°C and 36°C, separately.

SET	25°C			36°C			
	DNA						tRNA
	YO-PRO-1	YOYO-1			YO-PRO-1		
	K [×10 ⁶] [M ⁻¹]	K ₁ [×10 ⁷] [M ⁻¹]	K ₂ [×10 ⁵] [M ⁻¹]	K ₁ [×10 ⁷] [M ⁻¹]	K ₂ [×10 ⁴] [M ⁻¹]	K [×10 ⁵] [M ⁻¹]	K [×10 ⁵] [M ⁻¹]
1	0.9	3.1	3.3	0.5	3.4	5.0	6.9
2	2.0	3.5	1.2	2.0	5.0	9.8	7.8
3	1.0	3.2	1.9	2.3	6.9	7.6	7.7
4	0.7	4.0	2.8	2.3	-	-	-
5	1.1	2.7	1.4	-	-	-	-
6	1.2	3.5	1.7	-	-	-	-
AVG	1.1 ± 0.4	3.3 ± 0.4	2.0 ± 0.8	1.8 ± 0.8	5.1 ± 1.4	7.5 ± 2.0	7.5 ± 0.4

and the molecules' mobility patterns on the experimental data. These factors can significantly influence the observed fluorescence intensity and equilibrium constants.

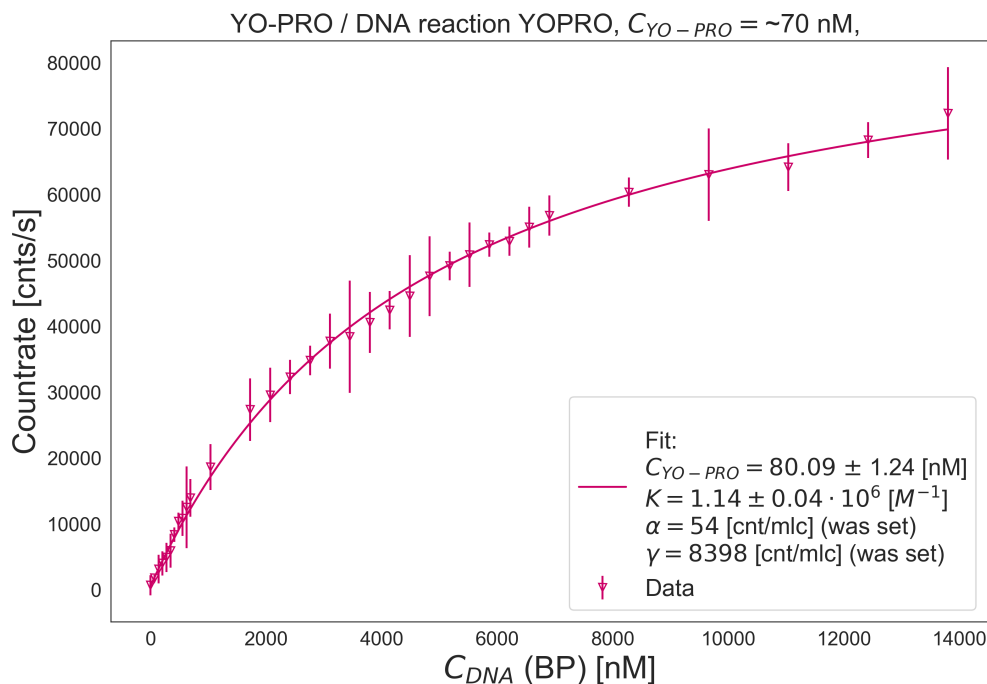


Fig. 27: Exemplary fit of equilibrium constant determination of DNA-YO-PRO-1 complex formation, at 25°C. Pink ▼ represents data points, indicating the count rate acquired for various DNA concentrations added to a 70 nM YO-PRO-1 solution in a titration experiment. The pink line corresponds to the fit of Equation (15). In the fit, C_{YO-PRO} represents the fitted value of the initial dye concentration, K is the equilibrium constant for the reaction, α is a molecular brightness of the free dye, and γ is a molecular brightness of the formed complex.

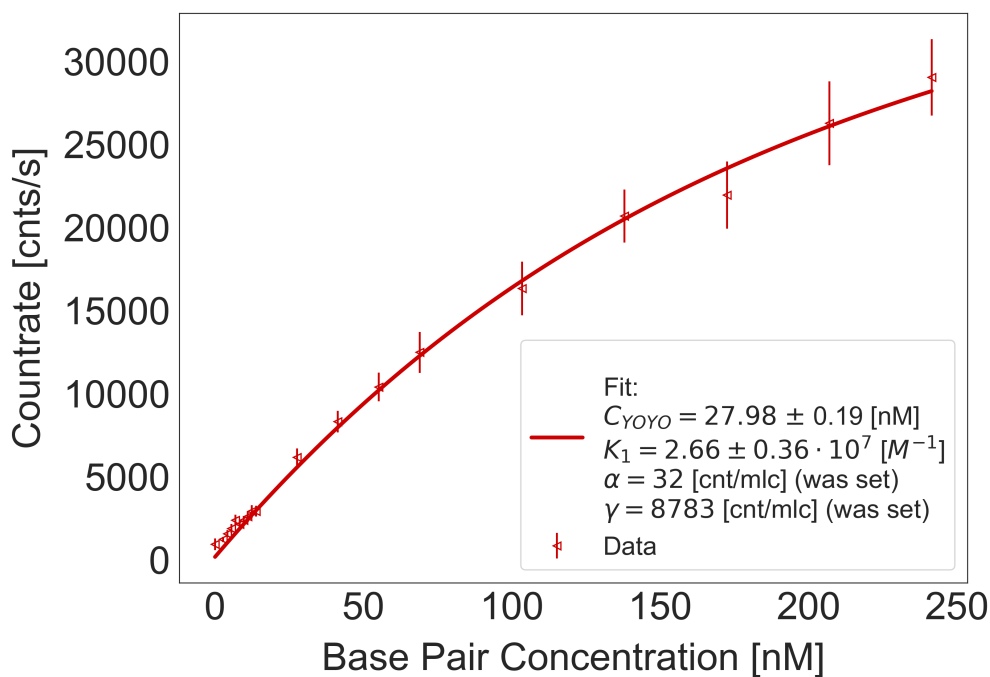


Fig. 28: Exemplary fit of equilibrium constant determination of DNA-YOYO-1 mono-intercalated complex formation, at 25°C, in equilibrium state. Red ▲ represents data points, indicating the count rate acquired for various DNA concentrations added to a 20 nM YOYO-1 solution in a titration experiment. The red line corresponds to the fit of Equation (15). In the fit, C_{YOYO} represents the fitted value of the initial dye concentration, and K_1 is the equilibrium constant for the mono-intercalation reaction.

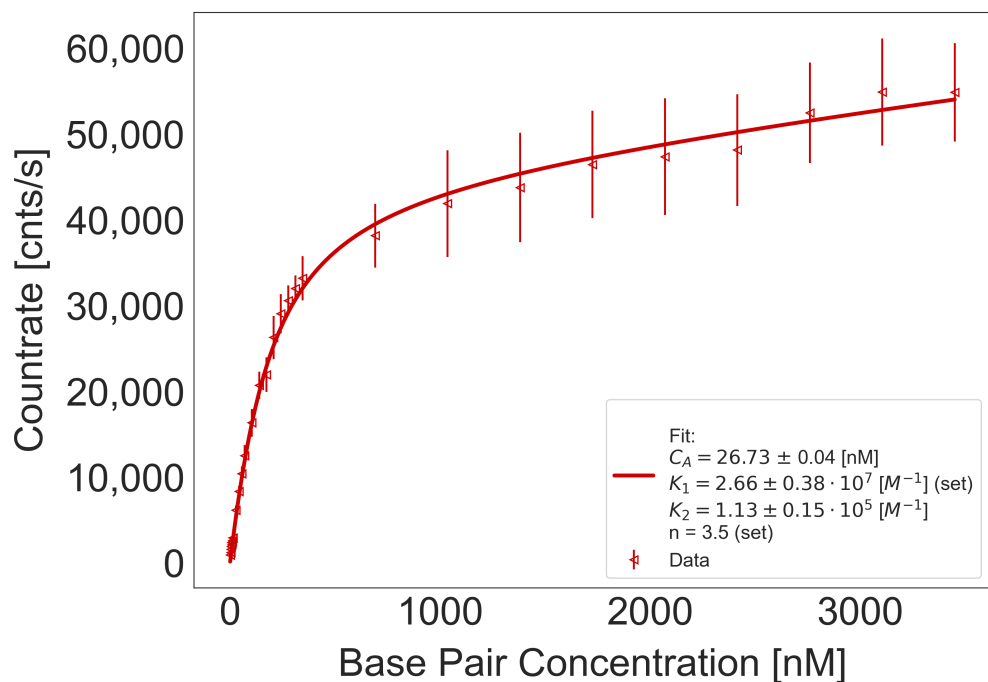


Fig. 29: Exemplary fit of equilibrium constant determination of DNA-YOYO-1 bis-intercalated complex formation, at 25°C, in equilibrium state. Red $\blacktriangle$ represents data points, indicating the count rate acquired for various DNA concentrations added to a 20 nM YOYO-1 solution in a titration experiment. The red line corresponds to the fit of Equation (20). In the fit, C_A represents the fitted value of the initial dye concentration, K_1 is the equilibrium constant for the mono-intercalation reaction (treated as constant), K_2 is the equilibrium constant for the bis-intercalation reaction, and n denotes the binding site size.

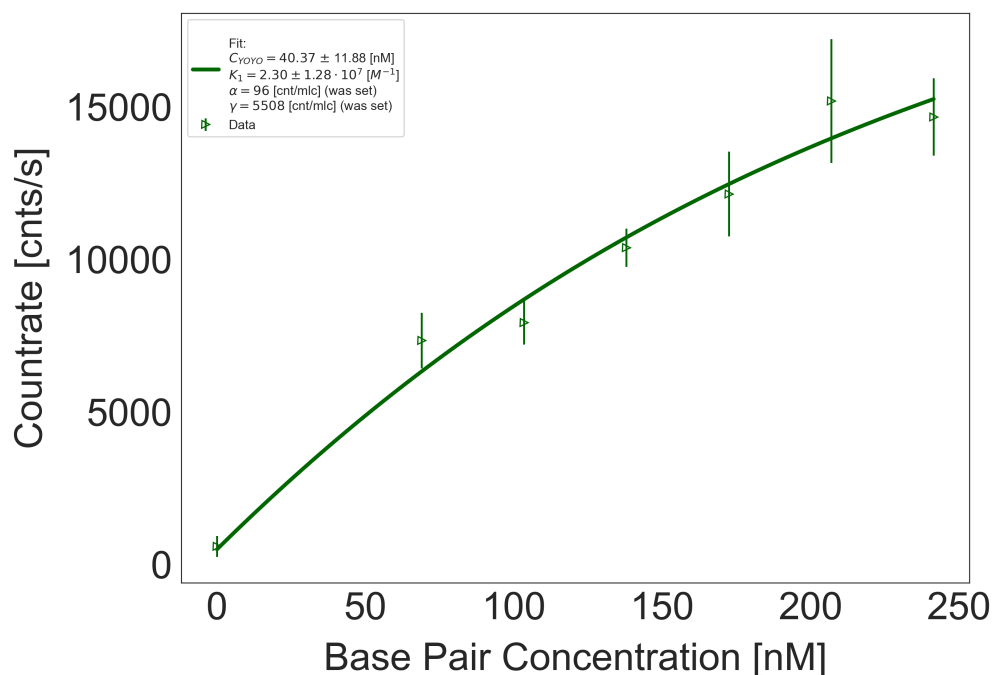


Fig. 30: Exemplary fit of equilibrium constant determination of DNA-YOYO-1 mono-intercalated complex formation, at 36°C, in equilibrium state. Green $\blacktriangleright$ represents data points, indicating the count rate acquired for various DNA concentrations added to a 40 nM YOYO-1 solution in a titration experiment. The green line corresponds to the fit of Equation (15). In the fit, C_{YOYO} represents the fitted value of the initial dye concentration, K_1 is the equilibrium constant for the mono-intercalation reaction, and n denotes the size of a binding site.

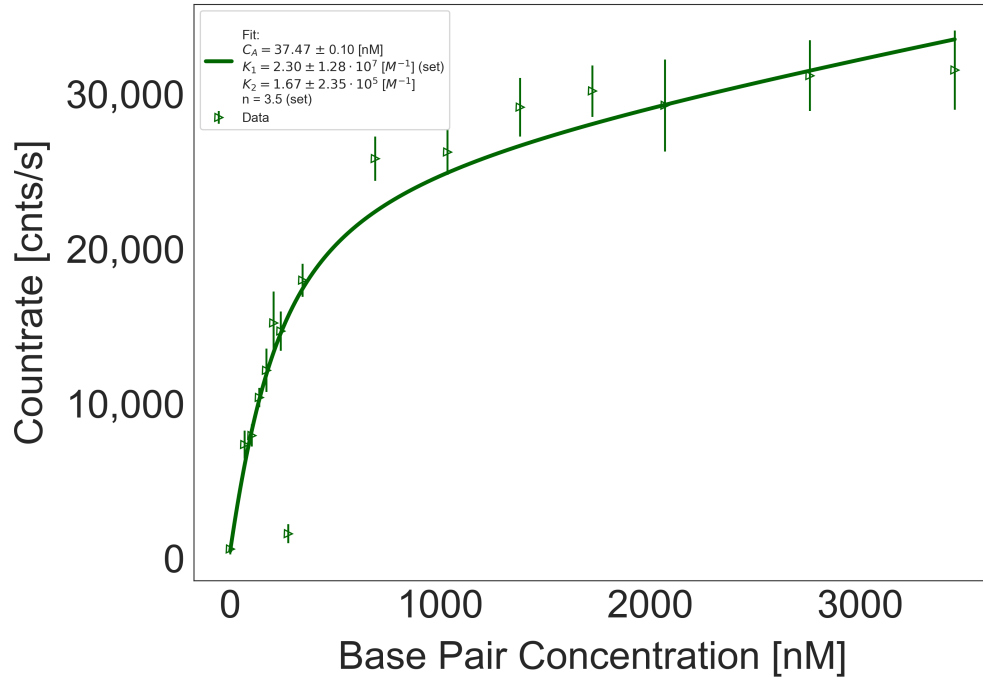


Fig. 31: Exemplary fit of equilibrium constant determination of DNA-YOYO-1 bis-intercalated complex formation, at 36°C, in equilibrium state. Green $\blacktriangleright$ represents data points, indicating the count rate acquired for various DNA concentrations added to a 40 nM YOYO-1 solution in a titration experiment. The green line corresponds to the fit of Equation (20). In the fit, C_A represents the fitted value of the initial dye concentration, K_1 is the equilibrium constant for the mono-intercalation reaction (treated as constant), K_2 is the equilibrium constant for the bis-intercalation reaction, and n denotes the binding site size.

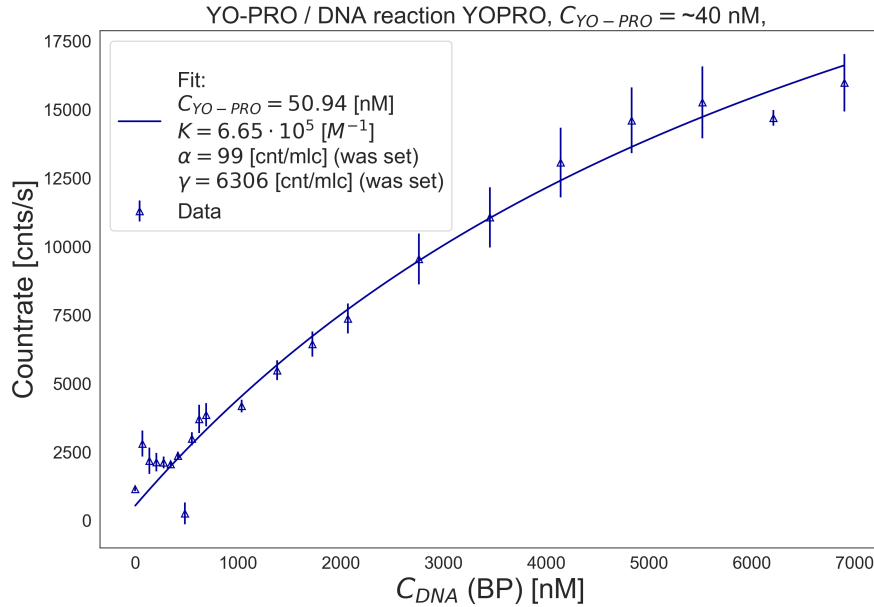


Fig. 32: Exemplary fit of equilibrium constant determination of DNA-YO-PRO-1 complex formation, at 36°C. Blue $\blacktriangle$ represents data points, indicating the count rate acquired for various DNA concentrations added to a 40 nM YO-PRO-1 solution in a titration experiment. The blue line corresponds to the fit of Equation (15). In the fit, C_{YO-PRO} represents the fitted value of the initial dye concentration, K is the equilibrium constant for the reaction, and α and γ denote the molecular brightnesses of the free dye and the formed complex, respectively.

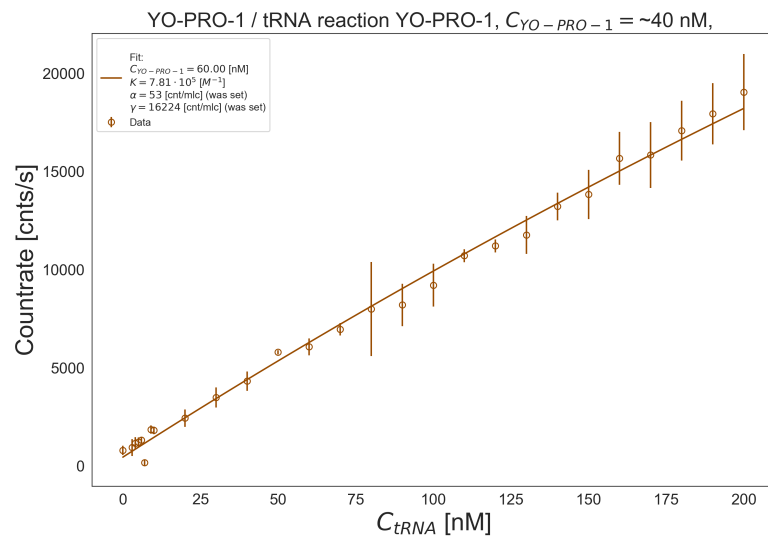


Fig. 33: Exemplary fit of equilibrium constant determination of tRNA-YO-PRO-1 complex formation, at 36°C, in equilibrium state. Brown circles represent data points showing the count rate for various tRNA concentrations added to a 40 nM YO-PRO-1 solution in a titration experiment. The brown line corresponds to the fit of Equation (15). In the fit, C_{YO-PRO} represents the fitted value of the initial dye concentration, and K is the equilibrium constant for the reaction. The data are calculated per concentration of molecules, while α and γ are the molecular brightnesses of the free dye and the formed complex, respectively.

Anthracyclines: doxorubicin, daunorubicin, idarubicin and epirubicin

For anthracycline reactions, as in the cyanine dye reactions, I first fitted the equilibrium constants using molecular brightnesses from consecutive experiments and then refitted the data using averaged molecular brightnesses.

Anthracyclines, in contrast to cyanine dyes, exhibit fluorescence properties in a free state. Moreover, as shown in the mechanism study, the fluorescence is quenched upon complex formation.

In Table 31, I collected molecular brightnesses and equilibrium constants obtained from all consecutive experiments of doxorubicin, daunorubicin, idarubicin and epirubicin with DNA (69 bp), performed at 36°C.

Based on the data collected in Table 31, unlike the cyanine dyes, anthracyclines inherently exhibit fluorescence properties in their free, unbound state.

This intrinsic fluorescence is a key feature that makes anthracyclines valuable for molecular and cellular imaging. However, an interesting observation from the experiments is that at elevated temperatures (36°C), the intrinsic fluorescence of the anthracyclines decreases [188].

This temperature-induced reduction in fluorescence suggests that the anthracyclines' molecular environment or structural conformation might change with temperature, influencing their fluorescence behaviour. Understanding these changes is essential for interpreting the interaction dynamics between anthracyclines and DNA and considering the potential impact of temperature on their use in research and therapeutic applications.

No consistent pattern was observed across all the anthracyclines for DRUG-DNA complex formation. Temperature had a different influence on the molecular brightness of the DRUG-DNA complexes. Specifically, an increase in temperature led to the rise in molecular brightness for doxorubicin and epirubicin complexes, suggesting enhanced fluorescence at elevated temperatures. This could indicate improved intercalation efficiency or altered electronic properties of these complexes at 36°C.

In contrast, the molecular brightness decreased with increasing temperature for daunorubicin and idarubicin complexes. This might reflect temperature-induced changes in the drug's interaction with DNA, possibly leading to less efficient intercalation or altered binding modes at higher temperatures.

These temperature-dependent trends were similarly observed for the (DRUG-DNA)-DRUG complexes, in which the molecular brightness varied with the anthracycline used, indicating that the secondary binding event also responds to temperature changes in a manner specific to each drug. Understanding these temperature-dependent behaviours is critical for interpreting the interactions between these anthracyclines and DNA and for assessing their potential implications for drug efficacy across different biological conditions.

The data were refitted by utilising averaged molecular brightnesses. Obtained equilibrium constants are collected in Table 32, while exemplary K_1 and K_2 fitting curves are shown in Figures 34 - 41.

The equilibrium constants for DRUG-DNA complex formation decreased by approximately one order of magnitude at 36°C compared to 25°C, indicating a temperature-dependent reduction in the binding affinity of the anthracyclines for DNA. This suggests that at higher temperatures, the DRUG-DNA interaction

may be slightly weakened, possibly due to altered conformational dynamics or disruption of favourable binding conditions.

In contrast, for the (DNA-DRUG)-DRUG complexes, the equilibrium constants decreased by two orders of magnitude at 36°C, indicating a more significant temperature-induced reduction in the stability of these secondary complexes. This suggests that the secondary binding event, in which the drug binds to the pre-formed DRUG-DNA complex, is more sensitive to temperature changes. However, daunorubicin showed an exception, where the equilibrium constant for the (DRUG-DNA)-DRUG complex formation decreased by only one order of magnitude at 36°C, suggesting that the temperature effect on the secondary binding event for daunorubicin is less pronounced compared to the other anthracyclines.

These findings highlight the varying temperature sensitivities of anthracyclines and their complexes with DNA, which could have implications for their use in cellular and therapeutic applications across different temperatures.

The results of these studies indicate that, in general, increased temperature decreases the molecular brightness of the studied complexes. However, this trend has some exceptions, which can be attributed to the chemical nature of the intercalators and their specific interactions with DNA. For instance, certain molecules, such as doxorubicin and epirubicin, exhibited increased molecular brightness with increasing temperature, whereas others, such as daunorubicin and idarubicin, showed a decrease. This variability underscores the complexity of temperature's impact on molecular interactions and highlights the need for a deeper understanding of these dynamics.

The observed temperature dependence of equilibrium constants — both in terms of molecular brightness and complex formation—suggests that further investigation is needed to uncover the underlying mechanisms responsible for these temperature effects. This could involve exploring the structural and electronic changes in the intercalators and DNA complexes at different temperatures.

Additionally, this research emphasises the importance of considering temperature effects on fluorescence when studying molecular interactions in vivo, particularly in cellular research, where temperature fluctuations can influence experimental outcomes. As fluorescence-based techniques become increasingly common in cellular and molecular biology, understanding the temperature dependence of fluorescence signals is essential for accurately interpreting experimental data.

Future studies on the complex formation of intercalators and other molecules with different reaction mechanisms could provide valuable insights into the role of temperature in fluorescence dynamics, ultimately enhancing the application of fluorophores in cellular research and improving the accuracy of fluorescence-based assays in various biological contexts.

Table 31: Compiled equilibrium constants and molecular brightnesses obtained from the analysed experimental data of the reactions between DNA and anthracyclines, carried out at 36°C, separately. Equilibrium constants for the reactions of DNA are calculated based on base-pair concentration.

SET	Doxorubicin				
	α [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ_1 [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ_2 [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	$K_1 [\times 10^6]$ [M ⁻¹]	$K_2 [\times 10^4]$ [M ⁻¹]
1	437	137	197	0.3	4.9
2	353	137	410	2.9	7.9
3	365	137	382	2.8	1.0
4	387	154	330	5.1	0.6
5	488	168	-	4.4	8.5
AVG	406 ± 50	147 ± 13	330 ± 82	3.1 ± 1.6	4.6 ± 3.3
SET	Daunorubicin				
	α [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ_1 [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ_2 [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	$K_1 [\times 10^6]$ [M ⁻¹]	$K_2 [\times 10^5]$ [M ⁻¹]
1	352	68	232	2.0	0.3
2	310	55	438	2.4	1.6
3	372	58	427	1.7	0.6
4	664	56	378	2.1	2.5
5	451	77	-	1.4	0.6
AVG	430 ± 126	63 ± 8	369 ± 82	1.9 ± 0.3	1.1 ± 0.8
SET	Idarubicin				
	α [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ_1 [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ_2 [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	$K_1 [\times 10^6]$ [M ⁻¹]	$K_2 [\times 10^4]$ [M ⁻¹]
1	135	19	174	2.9	4.4
2	92	14	340	3.1	3.5
3	93	17	257	4.6	3.1
4	-	-	204	-	-
AVG	107 ± 20	17 ± 2	244 ± 63	3.5 ± 0.7	3.7 ± 0.6
SET	Epirubicin				
	α [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ_1 [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	γ_2 [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]	$K_1 [\times 10^6]$ [M ⁻¹]	$K_2 [\times 10^4]$ [M ⁻¹]
1	390	242	204	2.6	4.4
2	354	243	441	1.7	3.5
3	365	249	440	2.6	3.1
4	-	-	365	-	-
AVG	370 ± 15	245 ± 3	363 ± 97	2.1 ± 0.7	4.0 ± 1.6

Table 32: Compiled equilibrium constants, fitted with averaged molecular brightnesses, of the reactions between DNA and anthracyclines, doxorubicin, daunorubicin, idarubicin and epirubicin, carried out at 36°C, separately.

SET	Doxorubicin		Daunorubicin		Idarubicin		Epirubicin	
	$K_1 [\times 10^6]$ [M ⁻¹]	$K_2 [\times 10^4]$ [M ⁻¹]	$K_1 [\times 10^6]$ [M ⁻¹]	$K_2 [\times 10^5]$ [M ⁻¹]	$K_1 [\times 10^6]$ [M ⁻¹]	$K_2 [\times 10^4]$ [M ⁻¹]	$K_1 [\times 10^6]$ [M ⁻¹]	$K_2 [\times 10^4]$ [M ⁻¹]
1	3.9	0.8	1.7	0.4	3.2	3.4	2.7	5.0
2	2.4	2.2	2.1	2.2	3.4	4.9	0.9	1.8
3	2.5	4.0	1.7	0.7	4.1	4.0	1.7	5.9
4	4.8	0.6	2.7	1.7	-	-	-	-
5	4.8	5.1	1.3	0.5	-	-	-	-
AVG	3.7 ± 1.0	2.5 ± 1.8	1.9 ± 0.5	1.1 ± 0.7	3.6 ± 0.4	4.1 ± 0.6	1.8 ± 0.8	4.2 ± 1.8

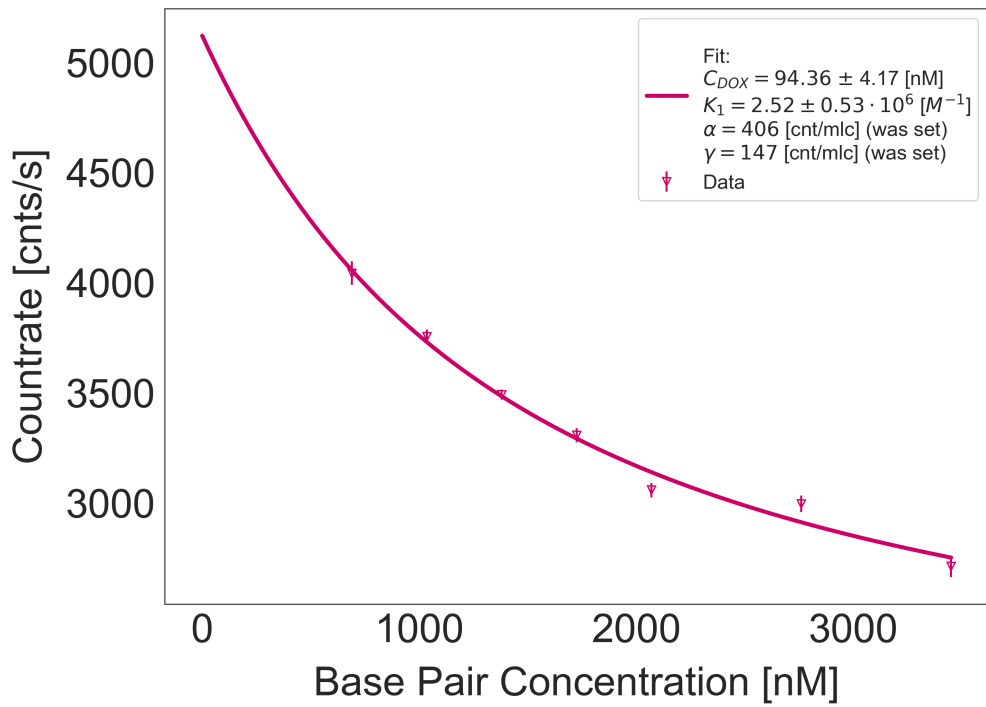


Fig. 34: Exemplary fit of equilibrium constant determination of DNA-DOX complex formation, at 36°C, in equilibrium state. Pink ▼ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM DOX solution in a titration experiment. The pink line corresponds to the fit of Equation (15). In the fit, C_{DOX} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction, while α and γ are molecular brightnesses of the free DOX and DNA-DOX complex, respectively.

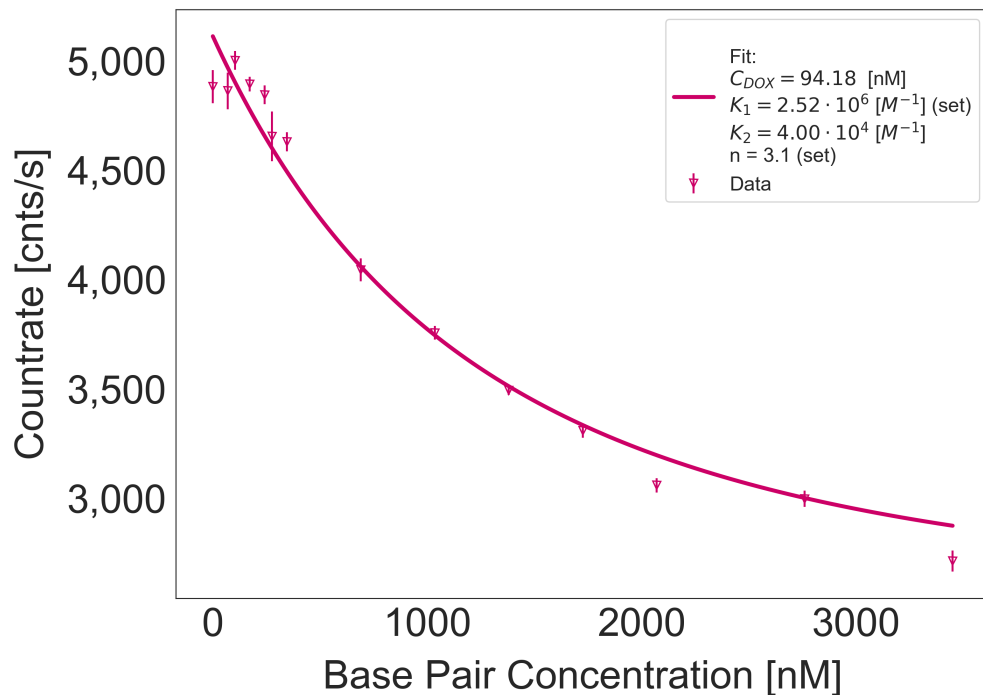


Fig. 35: Exemplary fit of equilibrium constant determination of (DNA-DOX)-DOX complex formation, at 36°C, in equilibrium state. Pink ▼ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM DOX solution in a titration experiment. The pink line corresponds to the fit of Equation (20). In the fit, C_A represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction (treated as constant), K_2 is the equilibrium constant for the (DNA-DOX)-DOX complex formation reaction, and n denotes the binding site size.

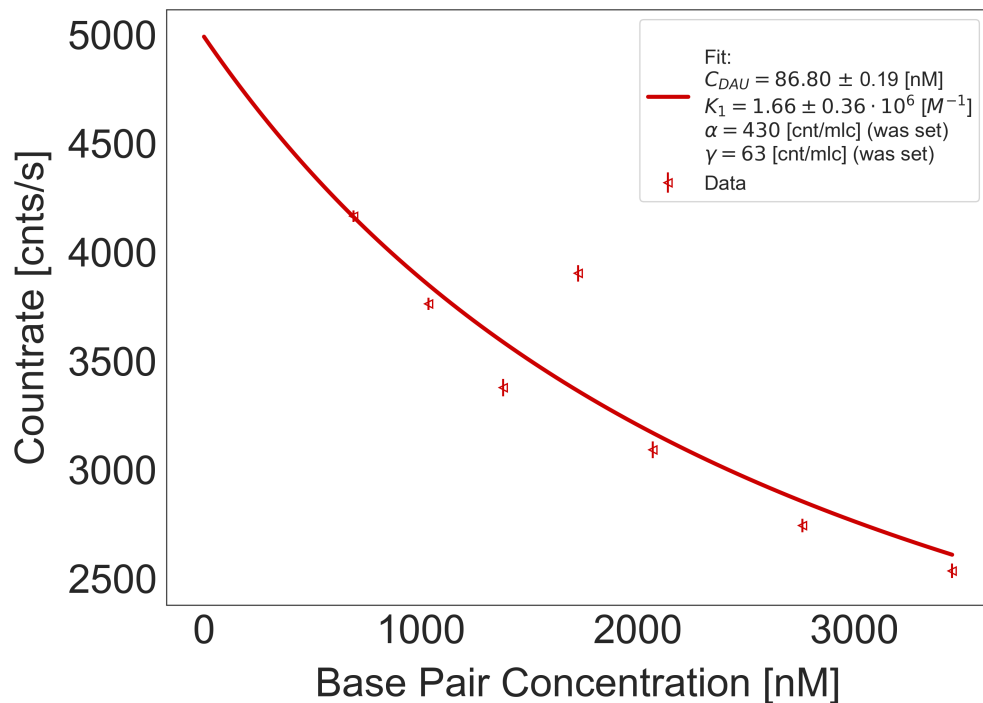


Fig. 36: Exemplary fit of equilibrium constant determination of DNA-DAU complex formation, at 36°C, in equilibrium state. Red ◄ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM DAU solution in a titration experiment. The red line corresponds to the fit of Equation (15). In the fit, C_{DAU} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction, while α and γ are molecular brightnesses of the free DAU and DNA-DAU complex, respectively.

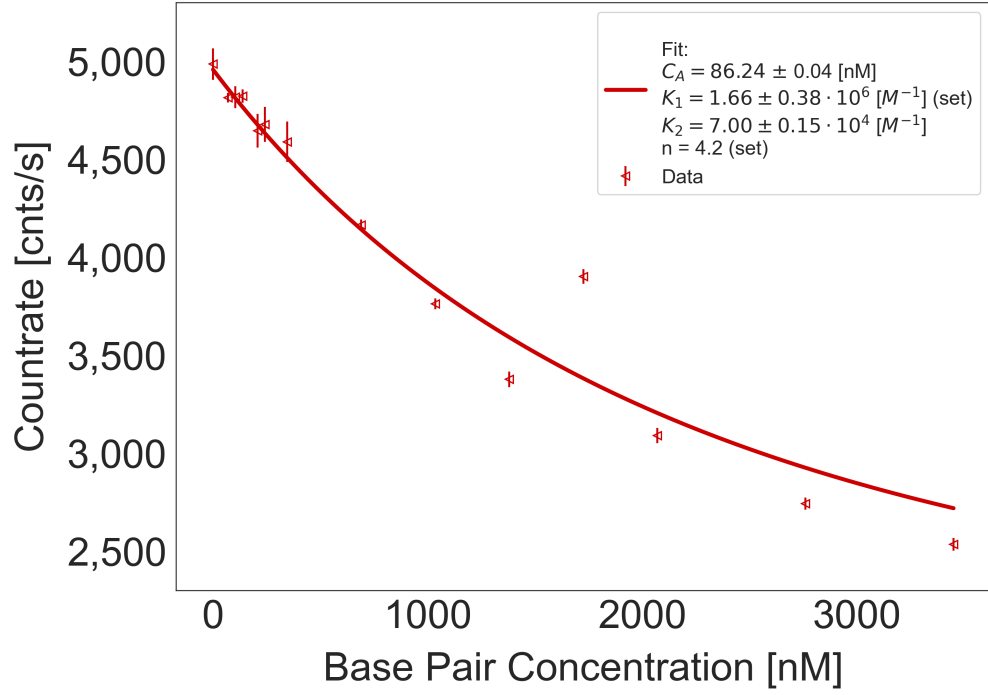


Fig. 37: Exemplary fit of equilibrium constant determination of (DNA-DAU)-DAU complex formation, at 36°C, in equilibrium state. Red $\blacktriangle$ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM DAU solution in a titration experiment. The red line corresponds to the fit of Equation (20). In the fit, C_A represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction (treated as constant), K_2 is the equilibrium constant for the (DNA-DAU)-DAU complex formation reaction, and n denotes the binding site size.

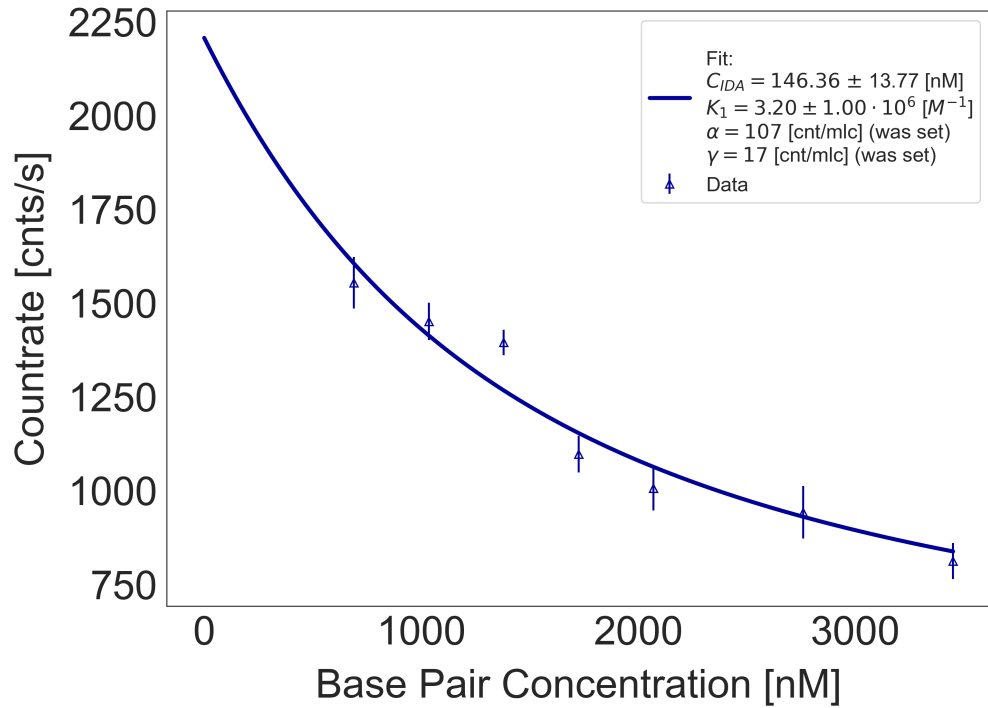


Fig. 38: Exemplary fit of equilibrium constant determination of DNA-IDA complex formation, at 36°C, in equilibrium state. Blue $\blacktriangle$ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM IDA solution in a titration experiment. The blue line corresponds to the fit of Equation (15). In the fit, C_{IDA} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction, while α and γ are molecular brightnesses of the free IDA and DNA-IDA complex, respectively.

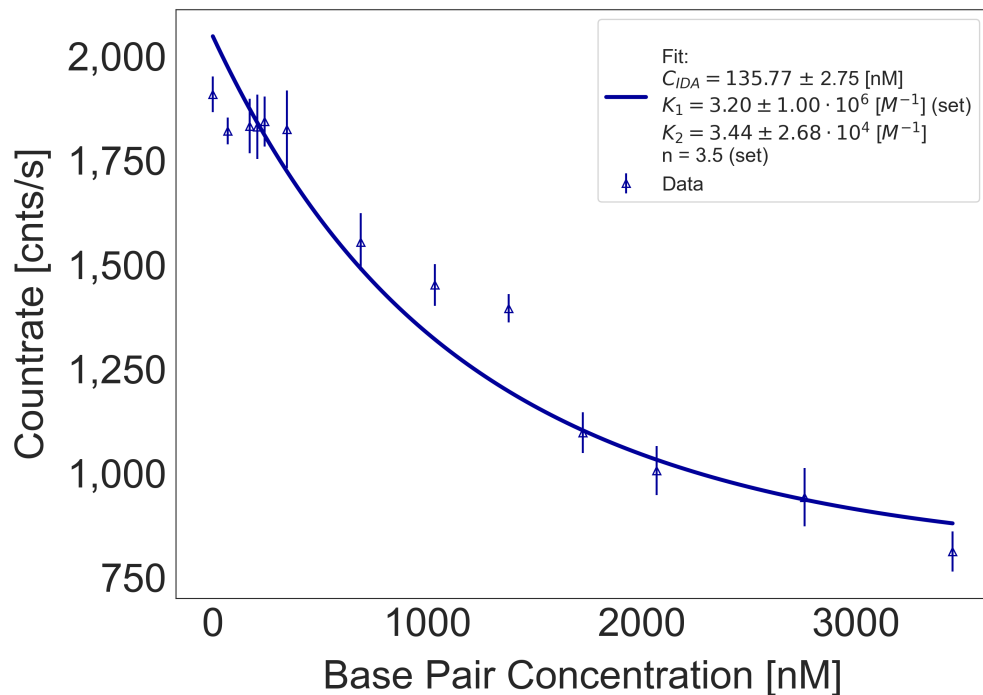


Fig. 39: Exemplary fit of equilibrium constant determination of (DNA-IDA)-IDA complex formation, at 36°C, in equilibrium state. Blue $\blacktriangle$ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM IDA solution in a titration experiment. The blue line corresponds to the fit of Equation (20). In the fit, C_{IDA} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction (treated as constant), K_2 is the equilibrium constant for the (DNA-IDA)-IDA complex formation reaction, and n denotes the binding site size.

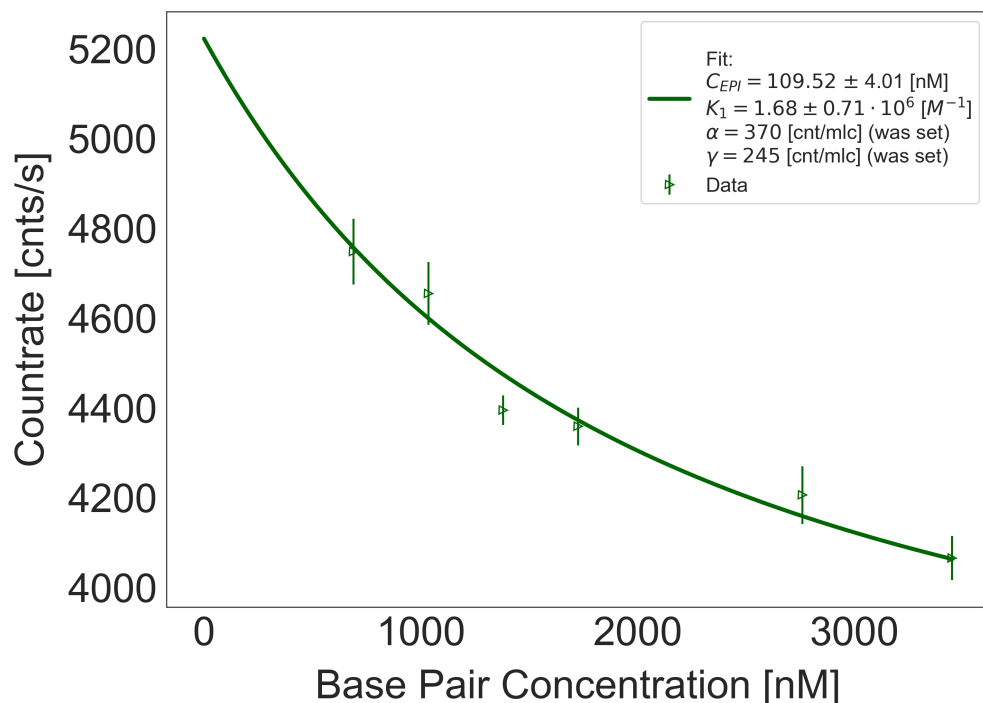


Fig. 40: Exemplary fit of equilibrium constant determination of DNA-EPI complex formation, at 36°C, in equilibrium state. Green $\blacktriangleright$ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM EPI solution in a titration experiment. The green line corresponds to the fit of Equation (15). In the fit, C_{EPI} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction, while α and γ are molecular brightnesses of the free EPI and DNA-EPI complex, respectively.

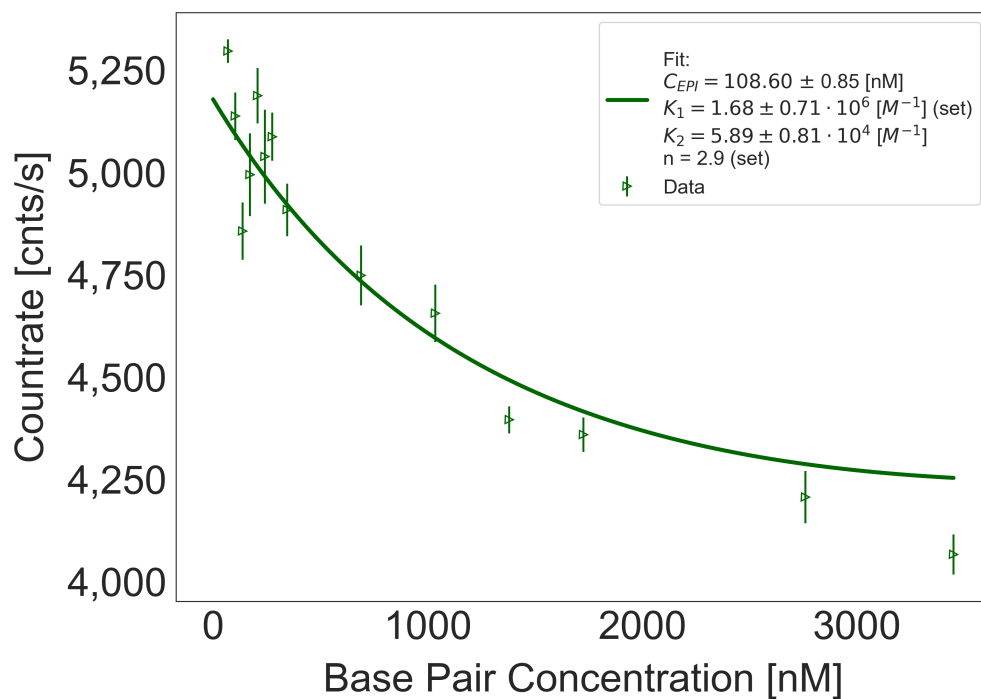


Fig. 41: Exemplary fit of equilibrium constant determination of (DNA-EPI)-EPI complex formation, at 36°C, in equilibrium state. Green $\blacktriangleright$ represents data points, indicating the count rate acquired for various DNA concentrations added to a 100 nM EPI solution in a titration experiment. The green line corresponds to the fit of Equation (20). In the fit, C_{EPI} represents the fitted value of the initial drug concentration, K_1 is the equilibrium constant for the first step of the complex formation reaction (treated as constant), K_2 is the equilibrium constant for the (DNA-EPI)-EPI complex formation reaction, and n denotes the binding site size.

3.2 Non-fluorescent complexes fraction determination

This section presents a study focusing on the immobilised DNA complexed with YO-PRO-1 dye and utilising continuous fluorescence photobleaching (CFP). Investigating immobilised structures is a critical step in understanding cellular processes, as it offers valuable insights into the behaviour of DNA when fixed in a controlled environment. These studies enable deeper exploration of molecular interactions and dynamics, which are often challenging to observe in living systems.

To achieve this, I meticulously combined Fluorescence Correlation Spectroscopy (FCS) — a technique typically used to analyse mobile probes — with confocal imaging. This precision-driven combination enabled exact positioning of the samples within the region of interest, ensuring that the fluorescence dynamics could be measured with high spatial resolution. By using this approach, I could accurately study the behaviour of immobilised DNA and its interactions with YO-PRO-1 dye, shedding light on the fluorescence characteristics of these complexes under well-defined experimental conditions.

Studying immobilised fluorescent particles presents several challenges, particularly due to potential biases in the detected fluorescence. Fluorescence is a phenomenon that relies on the absorption of energy, which excites the fluorophore and leads to emission. However, the ability of a fluorophore to absorb this energy is influenced by its orientation relative to the incident energy stream. When the fluorophore is immobilised, improper orientation can prevent it from effectively absorbing energy, resulting in no fluorescence.

This orientation issue means that some immobilised fluorophores might not contribute to the fluorescence signal, potentially leading to an underestimation of the total fluorescence. Consequently, not all reaction products may be captured in fluorescence measurements, leading to an incomplete representation of molecular interactions within the immobilised structure. Therefore, understanding and addressing this bias is crucial to ensuring the accuracy and reliability of the experimental results.

I performed a series of continuous fluorescence photobleaching experiments to address this issue. These experiments enabled me to determine the fraction of non-fluorescent complexes by monitoring the fluorescence from the emitting complexes over time. By comparing the observed fluorescence to the theoretical number of complexes expected in the focal volume, I could estimate the number of complexes that were not contributing to the fluorescence signal due to improper orientation.

This approach allowed me to meticulously account for the lack of fluorescence in specific immobilised complexes, effectively correcting for potential underrepresentation in the data. I obtained a more accurate representation of the total complex formation by subtracting the fluorescence signal from the non-emitting complexes. This thorough correction ensured that the results reflected the actual dynamics of the interaction between YO-PRO-1 dye and immobilised DNA.

To model eukaryotic nuclear DNA, I used phage λ DNA, which consists of 48,502 base pairs and adopts a spherical shape when immobilised. This choice of DNA was made to approximate the DNA found in a cell nucleus, enabling the study of molecular interactions in a controlled, simplified system. The spherical conformation of the immobilised λ DNA was particularly useful, as it closely re-

sembles the compacted DNA structure in specific cellular contexts, providing a relevant model for understanding DNA behaviour in the nucleus during the experiment.

To create a simplified model of DNA within the cell nucleus, I used a 5% agarose gel containing 30 pM DNA. To optimise the immobilisation of the DNA, I tested several gel formulations, including varying concentrations of agarose, gelatin, and a mixed agarose-gelatin gel (Fig. 42). The goal was to obtain immobilised DNA suspended in a transparent gel, ensuring the gel was well-homogenised and non-fluorescent, allowing for precise, accurate fluorescence measurements. Since the gelatin gel showed a fluorescence response, I used pure agarose gel.

Additionally, I explored a range of DNA concentrations (Fig. 43) from 10 to 50 pM to determine the optimal concentration for evenly distributing the immobilised DNA particles. Based on the distances between the centres of spherical DNA, I selected 30 pM as the final experiment concentration. This concentration provided a uniform distribution of DNA particles while maintaining an appropriate density, allowing clear visualisation of the DNA spheres in the imaging plane and ensuring the quality and reliability of the fluorescence data.

In summary, I immobilised phage λ DNA at 30 pM and saturated it with YO-PRO-1 dye in a 5% agarose gel to create a stable, well-homogenised system for subsequent fluorescence studies. This setup allowed for controlled observation of the interaction between the immobilised DNA and the YO-PRO-1 dye, providing valuable insights into the fluorescence dynamics and binding behaviour under the experimental conditions.

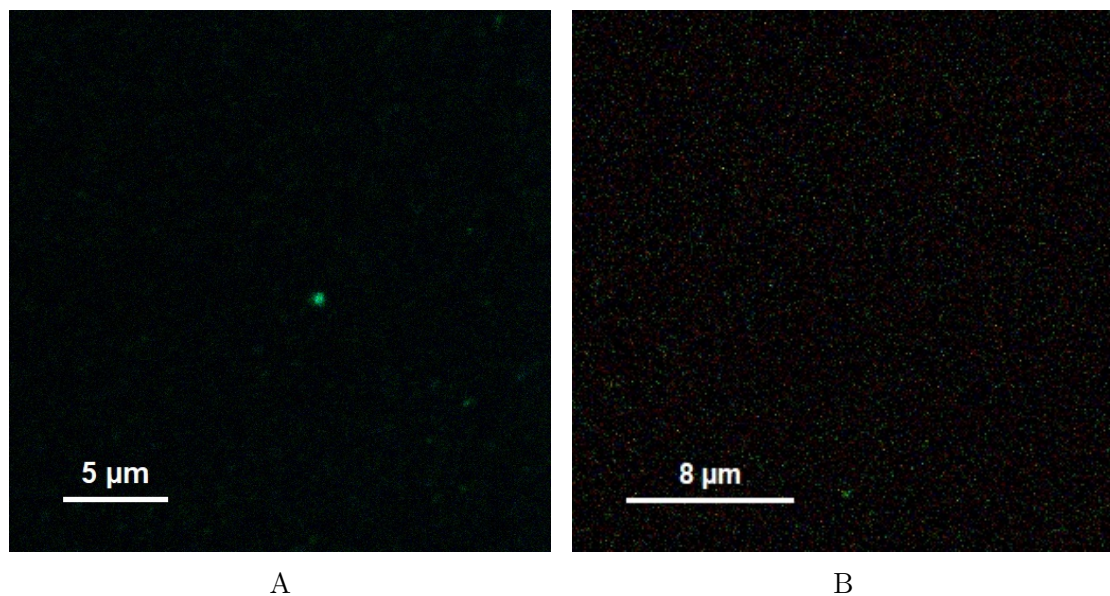


Fig. 42: The exemplary FLIM images of gels excited with a 485 nm laser. (A) FLIM image of gel containing 0.1% agarose and 6% gelatin. The bright, fluorescent spot of gelatin is observable. (B) FLIM image of gel containing 5% agarose. The gel does not give a fluorescence signal.

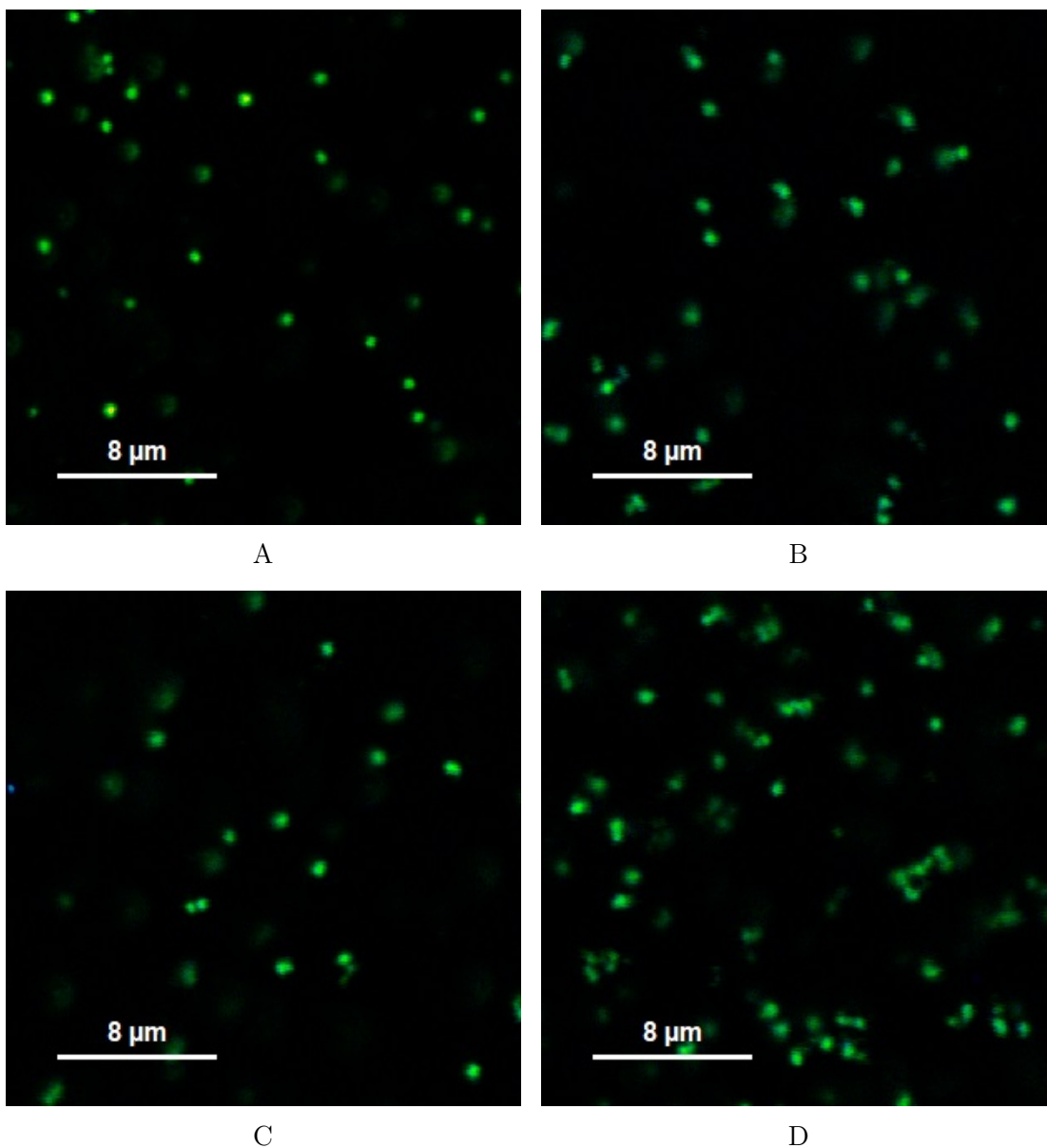


Fig. 43: Exemplary FLIM images of phage λ DNA stained with YOYO-1 dye immobilised in 5% agarose gel. Various concentrations of DNA were used: (A) 25 pM, (B) 30 pM, (C) 35 pM and (D) 40 pM. Distances between centres of spherical DNA were measured. Mean values were equal: (A) 2.63 μm , (B) 2.48 μm , (C) 1.84 μm and (D) 1.75 μm .

3.2.1 Determination of experimental and measurement parameters in 5% agarose gel

I tested linear and circular polarisation of the laser beam, used to photobleach YO-PRO-1 complexes with phage λ DNA. As standard, FCS applies a linearly polarised laser beam to illuminate the samples. I wanted to compare linear and circular polarisation and their effects on the photobleaching of fluorescence immobilised complexes (Fig. 20 in the Materials and Methods: Polarisation experiments section).

The main difference between standard FCS and the polarised FCS used was the elongated laser light pathway, which uses an additional optical fibre to connect the laser with the polarisation module and the polarisation module itself.

The polarisation module comprised two polarisation filters, allowing for linear and circular polarisation.

The experiments were performed at 25°C and 36°C to investigate the effect of temperature on the number of photobleached complexes.

To study Continuous Fluorescence Photobleaching (CFP), I combined Fluorescence Correlation Spectroscopy (FCS) with confocal imaging, allowing for high-resolution analysis of fluorescence dynamics. This combination enabled precise monitoring of the fluorophores while capturing detailed images of their distribution and behaviour within the system.

When studying phage λ DNA saturated with the bright fluorophore YO-PRO-1, the afterpulsing of the experimental setup must be considered. To eliminate afterpulsing from data analysis, I used two detectors and cross-correlated the recorded fluorescence.

I set up the system for fluorescence imaging to filter emissions within the 500-550 nm range.

Before the experiments, I first calibrated the equipment. The calibration was performed using buffer conditions and in a gel medium to accurately determine the focal volume's size. The calibration in the gel (Rhodamine 110 diffusing in a 5 % agarose gel) provided crucial information about the spatial dimensions of the focal volume, which directly influences the precision of the measurements.

Using the data from the gel calibration, I calculated the theoretical number of complexes present within the focal volume. This calculation is essential for understanding sample density and ensuring that the measurements reflect the actual concentration of the studied complexes. The values for the radius of the focal volume (ω) that were obtained from the gel calibration, performed at 25°C and 36°C, using both linear and circular polarisation, are summarised in Table 33, providing a reference for subsequent analyses and ensuring that the experimental setup was aligned correctly for optimal performance.

In each set of experiments, I utilised phage λ DNA saturated with YO-PRO-1 dye in PBS buffer. Phage λ DNA consists of 48,502 base pairs, leading to approximately 9,700 binding sites for the YO-PRO-1 dye, as the dye intercalates every five base pairs along the DNA strand. This intercalation property is crucial for accurately assessing the dynamics of dye and DNA binding.

As seen in Table 33, increased temperature leads to a widened focal volume. Notably, at 25°C, there was a nearly 20 nm difference between the linear and circular polarisation modes, with circular polarisation resulting in a larger radius. However, at 36°C, the difference in the width of the focal volume between the two polarisation modes became negligible.

This change can be attributed to thermal effects that influence the optical properties and geometry of the focal volume. At higher temperatures, the increase in the focal volume might result from altered behaviour of the optical setup, leading to more dispersed excitation conditions. The fact that the difference in focal volume size between the two polarisation modes diminished at elevated temperature could indicate that both linear and circular polarisation modes are similarly affected.

Table 33: Collected mean radiuses of the focal volume, obtained from calibration in the 5% agarose and calculated from all consecutive experiments, with applied linear or circular polarisation of the laser beam, carried out at 25°C and 36°C, separately.

SET	ω [nm]			
	Linear polarisation		Circular polarisation	
	25°C	36°C	25°C	36°C
1	241	276	205	247
2	269	292	298	276
3	231	264	270	292
4	220	262	241	259
5	222	278	-	251
6	-	270	-	283
7	-	258	-	278
8	-	-	-	270
9	-	-	-	258
AVG	237 ± 18	271 ± 11	254 ± 35	268 ± 14

Table 34: Values of fluorescence intensities collected from the saturated with YO-PRO-1 dye phage λ DNA in buffer conditions, with applied linear or polarisation of the laser beam, at 25°C or 36°C, separately.

SET	fluorescence intensity [$\frac{\text{kcnts}}{\text{s}}$]			
	Linear polarisation		Circular polarisation	
	25°C	36°C	25°C	36°C
1	224.3	783.7	388.8	578.6
2	296.5	309.2	278.2	977.6
3	351.7	982.8	190.9	517.8
4	372.9	1007.7	292.5	598.1
5	224.3	665.7	-	770.8
6	-	482.7	-	999.3
7	-	410.5	-	723.1
8	-	-	-	376.3
9	-	-	-	544.2
AVG	294.0 ± 62.1	663.2 ± 255.5	287.6 ± 70.2	676.2 ± 198.5

ted by temperature, likely because the broader excitation range provided by the circularly polarised beam becomes less relevant at higher temperatures.

This observation is important when considering the experimental conditions under which these measurements are made, especially when aiming to study fluorescent particles in complex systems such as DNA-probe interactions.

This widening of the focal volume at elevated temperatures may be attributed to thermal expansion of the microscope objective, which could contribute to variations in the optical volume parameters. Objectives and other optical components are subject to temperature-induced physical changes, which can affect the alignment and size of the focused laser spot.

When comparing the calibration data collected in buffer conditions (data not shown), a slight widening of the focal volume was also observed. However, this change remained within the margin of error.

Table 35: Molecular brightnesses of DNA-YO-PRO-1 complex obtained from all consecutive experiments, with applied linear or circular polarisation of the laser beam, at 25°C or 36°C, separately.

SET	Molecular brightness γ [$\frac{\text{cnts}}{\text{mol.} \cdot \text{s}}$]			
	Linear polarisation		Circular polarisation	
	25°C	36°C	25°C	36°C
1	46	81	80	60
2	31	32	57	101
3	36	101	40	53
4	38	104	60	62
5	38	69	-	79
6	-	50	-	103
7	-	42	-	75
8	-	-	-	39
9	-	-	-	56
AVG	38 ± 5	68 ± 26	59 ± 14	70 ± 20

I collected data from the DNA samples saturated with YO-PRO-1 in buffer to measure fluorescence intensity. The collected fluorescence intensity data for measurements performed at 25°C and 36°C, using both linear and circular polarisation, are presented in Table 34.

The difference in fluorescence intensity of the phage λ DNA saturated with YO-PRO-1 dye freely diffusing in PBS buffer was not observed when using linear and circular laser polarisation. However, higher temperatures increased the recorded fluorescence intensity.

I calculated the molecular brightness of the formed DNA-YO-PRO-1 complex in the buffer to determine the number of photobleached immobilised complexes. The MB values, γ , are summarised in the Table 35.

Except for the MB calculated for a linearly polarised laser used for DNA-YO-PRO-1 complex excitation, measured at 25°C, I did not observe an increase in molecular brightness with increasing temperature.

3.2.2 Determining the fraction of emitting DNA-YO-PRO-1 complexes immobilised in 5% agarose gel

Following the calibration and fluorescence intensity measurements, I proceeded with the continuous fluorescence photobleaching experiments.

During these experiments, I monitored the photobleaching of YO-PRO-1 complexes with phage λ DNA under the influence of a linearly or circularly polarised laser at 25°C or 36°C. First, I calculated the number of photobleached molecules using the molecular brightnesses from consecutive experiments. The data from these experiments, including the calculated decreases in fluorescence intensities and the corresponding number of photobleached complexes, are presented in Tables 36 - 39.

Next, I recalculated the number of photobleached molecules using the average molecular brightness values. By applying this average value to the photobleaching data, I could refine the calculation of the number of photobleached molecules, which accounts for potential variations in fluorescence intensity between individual

Table 36: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with molecular brightnesses from consecutive experiments and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied linear polarisation of the laser beam, at 25°C.

Fluorescence intensity [$\frac{\text{kcounts}}{\text{s}}$]	Number of molecules [$\times 10^3$]	Percentage [%]
57.6	1.3	60.34
55.4	1.2	57.99
59.6	1.9	74.34
54.5	1.8	67.87
48.9	1.6	60.98
41.9	1.4	52.20
44.2	1.4	55.14
41.0	1.3	51.08
45.0	1.5	56.15
36.1	1.0	52.62
32.2	0.9	46.92
52.8	1.4	80.26
52.9	1.4	80.48
61.4	1.6	93.27
53.1	1.4	80.74
35.4	0.9	52.84
26.5	0.7	39.60
45.8	1.2	68.31
Average	1.3 ± 0.3	62.84 ± 13.81

complexes.

Subsequently, I recalculated the percentage of visible DNA-YO-PRO-1 complexes based on the theoretical number of complexes in the focal volume. This refinement provides a more accurate estimate of the proportion of complexes that were effectively excited and contributed to the observed fluorescence signal during photobleaching.

The updated results for applying linear and circular polarisation of the laser at 25°C or 36°C, including the recalculated number of photobleached molecules and the percentage of visible complexes, are presented in Tables 40 - 43.

I observed more photobleached molecules at 36°C due to the larger focal volume. The increased focal volume at the higher temperature naturally led to more theoretical DNA-YO-PRO-1 complexes in the region of interest, thus increasing the potential for photobleaching.

Interestingly, despite the larger focal volume, more complexes were photobleached with linear polarisation than with circular polarisation. This can be explained by the distribution of energy in the laser beam. In the case of linear polarisation, the energy is more tightly focused in a specific direction, allowing for more efficient excitation of fluorophores in the focal volume. On the other hand, circular polarisation disperses the energy over a broader range, leading to a less concentrated excitation field. As a result, while circular polarisation excites a broader range of molecules, it does so less efficiently, resulting in fewer photobleached complexes. This highlights the importance of understanding the energy distribu-

Table 37: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with molecular brightnesses from consecutive experiments and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied circular polarisation of the laser beam, at 25°C.

Fluorescence intensity [$\frac{\text{kcnts}}{\text{s}}$]	Number of molecules [$\times 10^3$]	Percentage [%]
80.1	1.0	66.58
84.1	1.1	69.95
70.3	1.2	38.86
90.2	1.6	49.82
59.0	1.5	56.59
52.2	1.3	50.06
47.0	1.2	45.05
89.3	2.2	85.60
67.4	1.7	64.61
95.1	2.4	91.13
88.4	2.2	84.76
94.3	2.4	90.39
59.6	1.5	57.17
87.3	2.2	83.70
61.9	1.0	49.63
50.5	0.8	40.56
56.3	0.9	45.18
51.7	0.9	41.48
55.6	0.9	44.60
47.7	0.8	38.29
48.7	0.8	39.09
47.8	0.8	38.33
48.1	0.8	38.61
48.1	0.8	38.57
79.0	1.3	63.42
Average	1.3 ± 0.5	56.48 ± 17.96

tion of the laser beam in fluorescence experiments, as it significantly impacts the excitation efficiency and the resulting data.

When comparing the two laser polarisation modes, I observed that the ratio of photobleached complexes to the theoretical number of complexes was higher for linear polarisation, with a 10% increase compared to circular polarisation. This is consistent with linear polarisation's more efficient energy distribution, resulting in more fluorophores being excited and photobleached.

Interestingly, despite temperature-induced changes in focal volume size, molecular brightness, and the number of complexes, these effects cancel each other out. Specifically, while the increase in temperature led to a larger focal volume and higher molecular brightness, which would typically suggest more photobleaching, these factors were counteracted by the less efficient excitation with circular polarisation. As a result, no significant impact on the ratio of photobleached to the theoretical number of complexes was observed. This finding highlights the delicate interplay among experimental parameters, such as laser polarisation, temperature,

Table 38: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with molecular brightnesses from consecutive experiments and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied linear polarisation of the laser beam, at 36°C.

Fluorescence intensity [$\frac{\text{kcnts}}{\text{s}}$]	Number of molecules [$\times 10^3$]	Percentage [%]
126.2	1.6	57.19
90.5	1.1	41.00
97.6	1.2	44.22
86.0	1.1	38.94
169.0	2.1	76.56
94.7	3.0	97.07
69.1	2.2	70.79
79.0	2.5	80.92
50.5	1.6	51.70
132.4	1.3	52.58
119.1	1.2	47.30
130.2	1.3	51.69
137.6	1.4	54.65
142.3	1.4	56.51
138.0	1.3	54.04
166.5	1.6	65.22
133.2	1.3	52.16
141.9	1.4	55.59
133.0	1.3	52.08
160.6	2.3	84.19
79.9	1.2	41.88
136.7	2.0	71.67
98.8	2.0	75.78
95.0	1.9	72.84
88.6	1.8	67.96
88.1	1.8	67.54
65.0	1.5	65.01
71.4	1.7	71.39
76.5	1.8	76.45
74.6	1.8	74.55
88.7	2.1	88.73
Average	1.7 ± 0.4	63.17 ± 14.59

and focal volume, and emphasises the importance of considering these factors in fluorescence experiments.

Summarising the data in Tables 36 - 39, I found that approximately 50-60% of the immobilised complexes were photobleached, while 40-50% of the complexes remained non-excited during fluorescence measurements. This finding underscores a crucial consideration for standard Fluorescence Correlation Spectroscopy (FCS) experiments with immobilised setups. Specifically, when studying immobilised fluorophores, about 40% of the molecules may not contribute to the fluorescence signal due to incorrect orientation or other factors affecting their ability to absorb

energy and emit fluorescence.

This factor must be carefully considered in future analyses, as it can lead to underestimation of the number of active fluorophores in the system. The results emphasise the need for precise calibration and thorough understanding of the system's limitations when concluding fluorescence-based studies, particularly when dealing with immobilised molecular complexes.

Table 39: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with molecular brightnesses from consecutive experiments and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied circular polarisation of the laser beam, at 36°C.

Fluorescence intensity [$\frac{\text{kcnts}}{\text{s}}$]	Number of molecules [$\times 10^3$]	Percentage [%]
61.3	1.0	46.79
79.4	1.3	60.83
53.5	0.9	40.83
92.6	0.9	33.64
72.8	0.7	26.45
87.0	0.9	31.62
95.0	0.9	34.51
58.2	1.1	36.00
73.1	1.4	45.24
70.1	1.3	43.39
74.9	1.4	46.34
68.6	1.1	46.12
73.1	1.2	49.17
102.1	1.6	68.66
65.7	1.1	44.17
57.3	0.9	38.54
64.0	1.0	43.04
91.1	1.5	61.24
78.0	1.3	52.41
112.0	1.4	62.94
120.2	1.5	67.56
111.5	1.4	62.67
121.9	1.5	68.48
140.6	1.4	47.64
129.7	1.3	43.95
159.0	1.5	53.89
129.0	1.3	43.71
135.7	1.8	65.46
142.7	1.9	68.85
180.2	2.4	86.94
141.9	1.9	68.44
94.2	2.4	92.58
84.9	2.2	83.51
85.6	2.2	84.18
81.4	2.1	80.07
70.1	1.8	68.93
54.7	1.0	41.00
63.7	1.1	47.80
69.9	1.2	52.43
84.1	1.5	63.10
81.8	1.5	61.38
Average	1.4 ± 0.4	55.23 ± 16.09

Table 40: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with averaged molecular brightness and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied linear polarisation of the laser beam, at 25°C.

Fluorescence intensity [$\frac{\text{kcnts}}{\text{s}}$]	Number of molecules [$\times 10^3$]	Percentage [%]
57.6	1.5	73.42
55.4	1.5	70.57
59.6	1.6	60.97
54.5	1.4	55.66
48.9	1.3	50.01
41.9	1.1	42.81
44.2	1.2	45.22
41.0	1.1	41.89
45.0	1.2	46.05
36.1	1.0	50.12
32.2	0.9	44.69
52.8	1.4	80.68
52.9	1.4	80.91
61.4	1.6	93.77
53.1	1.4	81.17
35.4	0.9	53.12
26.5	0.7	39.81
45.8	1.2	68.67
Average	1.2 ± 0.3	59.97 ± 16.27

Table 41: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with averaged molecular brightness and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied circular polarisation of the laser beam, at 25°C.

Fluorescence intensity [$\frac{\text{kcnts}}{\text{s}}$]	Number of molecules [$\times 10^3$]	Percentage [%]
80.1	1.4	89.90
84.1	1.4	94.45
70.3	1.2	37.38
90.2	1.5	47.92
59.0	1.0	38.21
52.2	0.9	33.80
47.0	0.8	30.41
89.3	1.5	57.79
67.4	1.1	43.62
95.1	1.6	61.52
88.4	1.5	57.22
94.3	1.6	61.03
59.6	1.0	38.59
87.3	1.5	56.51
61.9	1.0	50.26
50.5	0.9	41.08
56.3	1.0	45.76
51.7	0.9	42.00
55.6	0.9	45.17
47.7	0.8	38.77
48.7	0.8	39.59
47.8	0.8	38.82
48.1	0.8	39.10
48.1	0.8	39.06
79.0	1.3	64.23
Average	1.1 ± 0.3	49.29 ± 15.58

Table 42: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with averaged molecular brightness and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied linear polarisation of the laser beam, at 36°C.

Fluorescence intensity [$\frac{\text{kcnts}}{\text{s}}$]	Number of molecules [$\times 10^3$]	Percentage [%]
126.2	1.8	67.69
90.5	1.3	48.54
97.6	1.4	52.35
86.0	1.3	46.10
169.0	2.5	90.63
94.7	1.4	45.39
69.1	1.0	33.10
79.0	1.2	37.84
50.5	0.7	24.18
132.4	1.9	77.61
119.1	1.7	69.81
130.2	1.9	76.30
137.6	2.0	80.67
142.3	2.1	83.41
138.0	2.0	82.13
166.5	2.4	99.12
133.2	1.9	79.28
141.9	2.1	84.48
133.0	1.9	79.15
160.6	2.3	84.89
79.9	1.2	42.23
136.7	2.0	72.27
98.8	1.4	55.37
95.0	1.4	53.22
88.6	1.3	49.65
88.1	1.3	49.35
65.0	1.0	39.90
71.4	1.0	43.82
76.5	1.1	46.92
74.6	1.1	45.76
88.7	1.3	54.46
Average	1.6 ± 0.5	61.15 ± 19.26

Table 43: Calculated decrease of fluorescence intensity in continuous fluorescence photobleaching experiments, number of photobleached molecules calculated with averaged molecular brightness and percentage of visible complexes in theoretical number of complexes in the focal volume, with applied circular polarisation of the laser beam, at 36°C.

Fluorescence intensity [$\frac{\text{kcnts}}{\text{s}}$]	Number of molecules [$\times 10^3$]	Percentage [%]
61.3	0.9	40.23
79.6	1.1	52.30
53.5	0.8	35.11
92.6	1.3	48.70
72.8	1.0	38.29
87.0	1.2	45.77
95.0	1.4	49.96
58.2	0.8	27.34
73.1	1.0	34.36
70.1	1.0	32.96
74.9	1.1	35.20
68.6	1.0	40.98
73.1	1.0	43.69
102.1	1.5	61.01
65.7	0.9	39.25
57.3	0.8	34.24
64.0	0.9	38.24
91.1	1.3	54.41
78.0	1.1	46.57
112.0	1.6	71.25
120.0	1.7	76.49
111.5	1.6	70.95
121.9	1.7	77.53
140.6	2.0	70.31
129.7	1.9	64.88
159.0	2.3	79.55
129.0	1.8	64.52
135.7	1.9	70.36
142.7	2.0	74.00
180.2	2.6	93.44
141.9	2.0	73.56
94.2	1.3	51.74
84.9	1.2	46.68
85.6	1.2	47.05
81.4	1.2	44.75
70.1	1.0	38.53
54.7	0.8	32.91
63.7	0.9	38.36
69.9	1.0	42.08
84.1	1.2	50.64
81.8	1.2	49.26
Average	1.3 ± 0.4	51.89 ± 16.04

3.3 YO-PRO-1 dye targets determination

In this chapter, I present my investigation on YO-PRO-1 dye cellular targets. The dye is primarily known to intercalate into double-stranded DNA.

I stained HeLa cells with YO-PRO-1 dye solution (Fig. 44). Despite the fact that YO-PRO-1 dye is considered a double-stranded DNA stain, I observed staining of the cytoplasm volume, prominent staining of nucleoli, and the nuclear membrane. Interestingly, I did not observe nuclear DNA staining.

The intensive staining of nucleoli was unexpected. Nucleoli are specialised nuclear compartments, in which ribosome biogenesis takes place [88]. This might indicate that ribosomes or their subunits are a potential YO-PRO-1 cellular target. It could also explain the cytoplasm and nuclear membrane staining, as ribosomes are present in both localisations.

To confirm my assumptions, I tested the reaction with YO-PRO-1 dye using various nucleic acids and molecules in the buffer. I expected an increase in fluorescence, as in the reaction between double-stranded DNA and the dye. Examined molecules included various nucleic acids (mRNA, DNA hairpins, and ssDNA) and the HSA protein, the most abundant protein in cells. As a control, I also measured

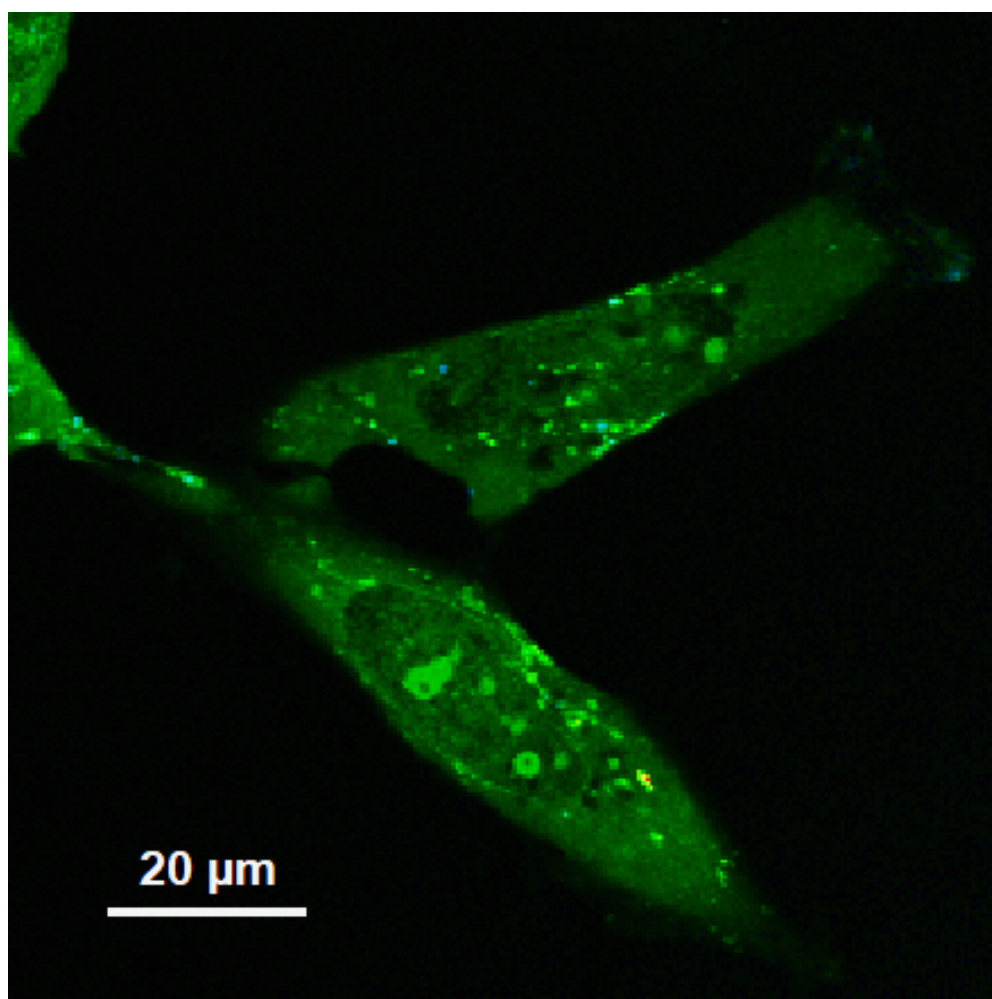


Fig. 44: Confocal image of HeLa cells stained with YO-PRO-1 dye. In the image, two cells are visible. Nucleoli, the nuclear membrane, and the cytoplasm are stained with the dye. HeLa cells were incubated with 50 nM YO-PRO-1 dye for one hour.

Table 44: Fluorescence intensities of the nucleic acid samples without or with YO-PRO-1 dye, at 36°C.

Nucleic Acid	Countrate without dye	Countrate with	Reaction
ssDNA	545	644	NO
HSA	6066	6869	NO
hairpin	410	671	NO
mRNA	552	657	NO
Cell lysate	1503	2747	YES
Cell lysate with RNase	3128	3860	YES

the molecules without adding the dye. Collected intensities from these experiments are collected in Table 44.

I expected YO-PRO-1 to react with hairpins, as they contain double-stranded regions; however, no reaction was observed. The most likely explanation for the absence of interaction is the short half-life of these double-stranded segments.

I was unable to identify YO-PRO-1 cellular targets via buffer tests; therefore, I tested HeLa cell lysates. Lysates were prepared with a non-denaturing buffer and contain DNA and a mixture of RNAs, including rRNA, tRNA, mRNA, and mitochondrial RNA. Given my suspicion, based on the HeLa cells imaging with prominently stained nucleoli (Fig. 44), that the dye interacts with RNA — particularly rRNA and tRNA, which contain double-stranded regions — I treated one of the lysate samples with RNase to verify whether using the enzyme would result in any detectable difference.

I observed the reaction of YO-PRO-1 with the lysate components, and recorded an increase in fluorescence and autocorrelation function (Fig. 45). However, I could not fit the autocorrelation curves due to the presence of numerous fluorescent species in the samples.

In the RNase-treated sample, the fluorescence count rate increase was lower compared to the untreated sample (Table 44, Fig. 46). This effect can be explained by RNase digestion, which cleaves RNA molecules into shorter fragments, thereby raising the number of fluorescent species within the focal volume.

As an extension of the observation that YO-PRO-1 binds RNA, I next measured the total RNA pool to further investigate its interaction with the dye. The data obtained were fitted using a two-component diffusion model with rotational diffusion of the bigger component (Fig. 47).

The first component, which had a small size with a 2.1 nm radius, exhibited a diffusion coefficient of $153 \mu\text{m}^2/\text{s}$ and a concentration of 5.3 nM. Based on these parameters, this component was likely tRNA. The second, larger component had a 24.5 nm radius, a diffusion coefficient of $12.1 \mu\text{m}^2/\text{s}$, and a concentration of 8.5 nM. These characteristics suggested that this component was ribosomes, which contain rRNA and ribosomal proteins, confirming that YO-PRO-1 binds to both tRNA and rRNA within the cell, in addition to its interaction with DNA. [?]

To further validate the interaction between YO-PRO-1 and RNA, I measured tRNA and the YO-PRO-1 dye mixture. As expected, the data could be fitted with a one-component diffusion model, consistent with the behaviour of a single type of molecule. The results indicated that tRNA is a cellular target of the YO-PRO-1 dye. The calculated parameters for tRNA were a radius of 1.9 nm, a diffusion

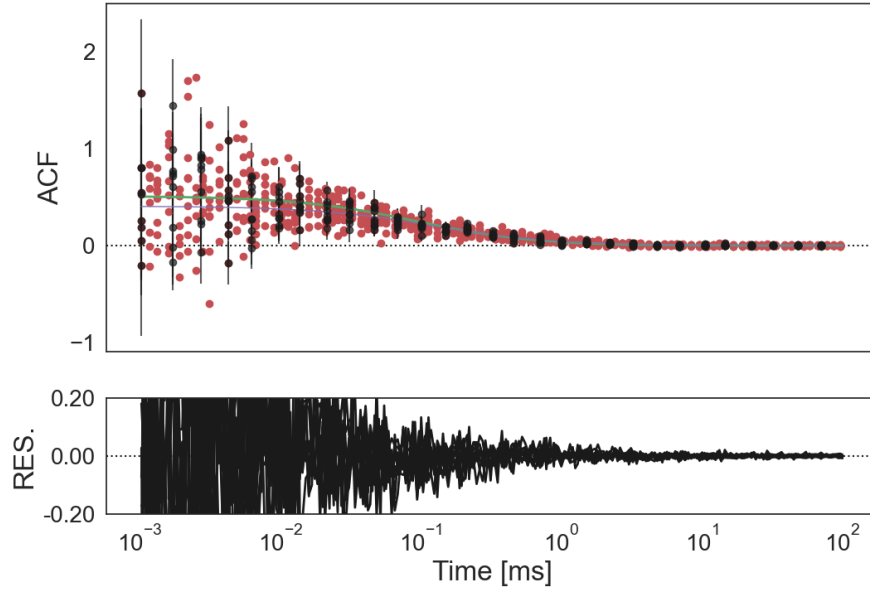


Fig. 45: Autocorrelation obtained from the fitting of FCS measurement of the cell lysate with YO-PRO-1 dye. Red and black (every tenth) points are experimental data (9 separate measurements), green line is the fitting of a one-component diffusion model, and violet line is the autocorrelation based on the averaged parameters.

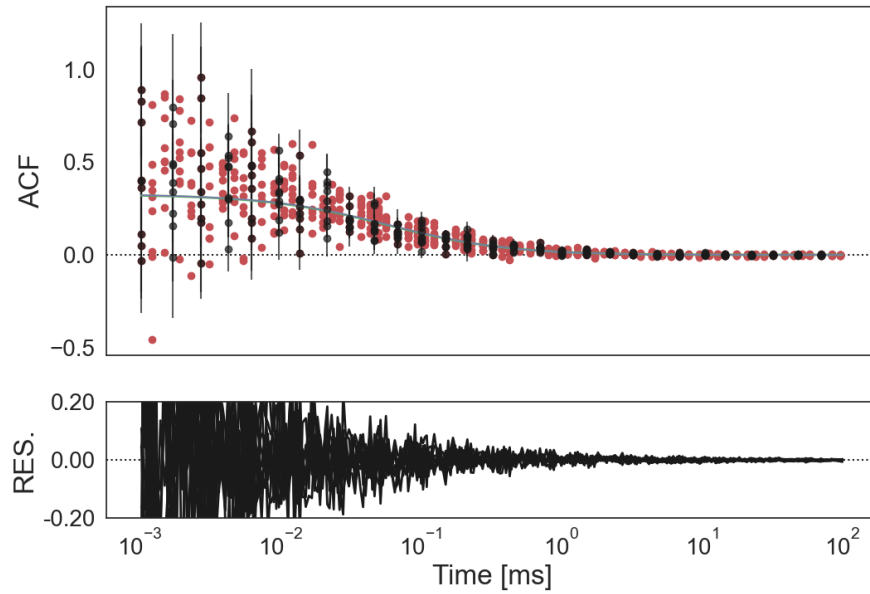


Fig. 46: Autocorrelation obtained from the fitting of FCS measurement of the cell lysate treated with RNase, with YO-PRO-1 dye. Red and black (every tenth) points are experimental data (9 separate measurements), green line is the fitting of a one-component diffusion model, and violet line is the autocorrelation based on the averaged parameters.

coefficient of $167 \mu\text{m}^2/\text{s}$, and a concentration of 39.3 nM . These findings confirmed that YO-PRO-1 binds to tRNA within the cellular environment (Fig. 48).

Finally, it was confirmed that the large ribosomal subunit (LSU) is another potential cellular target of YO-PRO-1. This extension of the study focused on exa-

mining the translational and rotational diffusion of the LSU, building on previous research regarding YO-PRO-1's cellular targets [?]. The findings further expand our understanding of the dye's interactions in a cellular context.

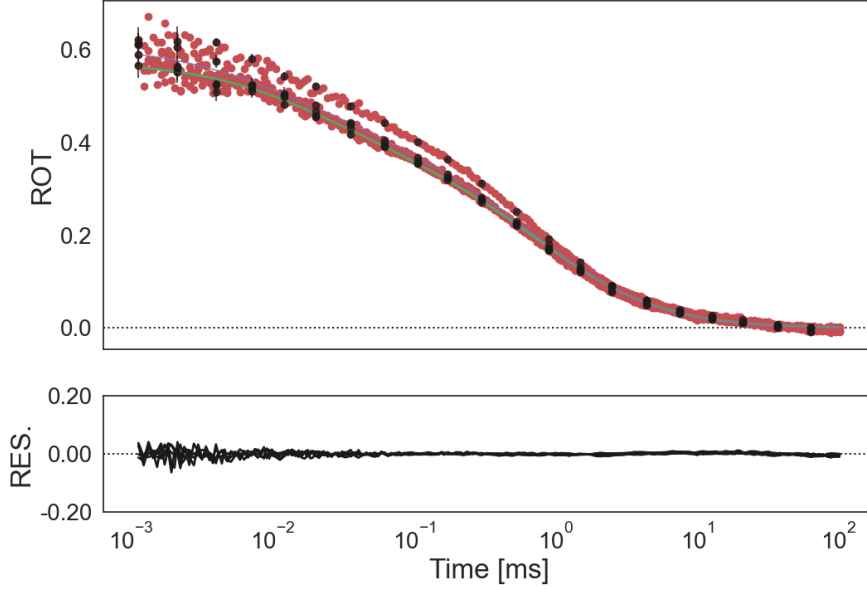


Fig. 47: Autocorrelation obtained from the fitting of FCS measurement of the total pool of RNA, with YO-PRO-1 dye. Red and black (every tenth) points are experimental data, green line is the fitting of a two-component diffusion model with rotational diffusion of the bigger component, and violet line is the autocorrelation based on the averaged parameters.

Obtained values of the hydrodynamic radii for tRNA and ribosomes are in good correlation with literature data (Tables 1 and 2).

My studies have identified several critical cellular targets for YO-PRO-1, including tRNA, rRNA, and the LSU. These findings offer valuable insights into the molecular interactions of YO-PRO-1 within the cellular environment and contribute to a more comprehensive understanding of its behaviour in biological systems.

The project was divided into *in vitro* and *in vivo* measurements. I was responsible for the *in vitro* part of the study, while the *in vivo* data are included in my colleague's dissertation.

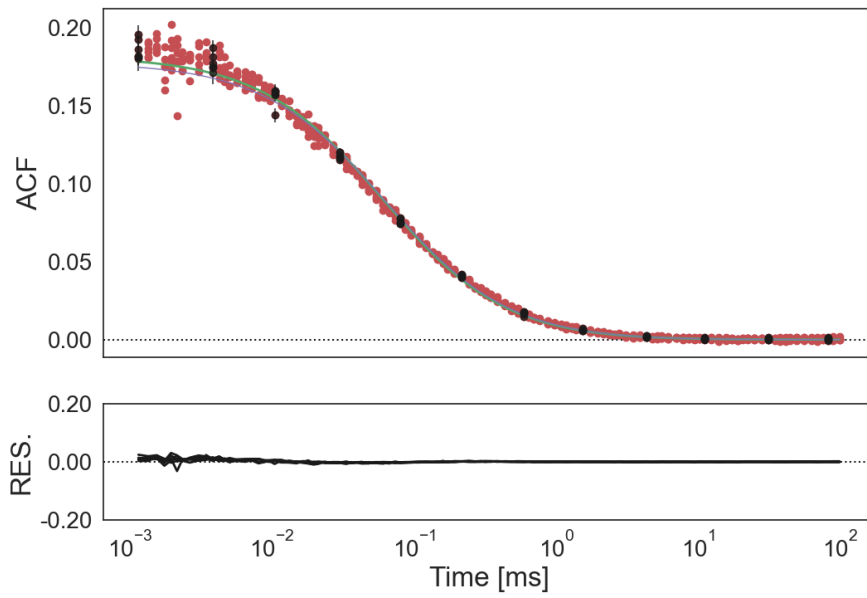


Fig. 48: Autocorrelation obtained from the fitting of FCS measurement of the tRNA, with YO-PRO-1 dye. The red and black (every tenth) points are experimental data, the green line is the fitting of a one-component diffusion model, and the violet line is the autocorrelation based on the averaged parameters.

4 Main results, conclusions and future prospects

Cyanine dyes equilibrium constants

Nucleic acids are among the most fundamental biological molecules, and even subtle disturbances in their structure can influence essential cellular processes such as replication, transcription, translation, and gene regulation. Fluorescent dyes are widely used as highly sensitive probes for studying DNA and RNA structure, dynamics, localisation, and function both in vitro and in cellular systems. Their strong fluorescence enhancement upon binding to nucleic acids makes them particularly useful in fluorescence microscopy, flow cytometry, and quantitative biochemical assays.

However, fluorescent probes should not be regarded as fully inert labels. Intercalating dyes, such as the cyanine dyes YO-PRO-1 and YOYO-1, can modify the local structure and dynamics of nucleic acids by inserting between stacked bases. These structural perturbations may affect the biological functions of DNA and RNA and, at the same time, provide valuable information about the limits of nucleic acid structural flexibility. In this context, kinetic and equilibrium studies of dye–nucleic acid interactions are essential for understanding not only the properties of the dyes themselves, but also the structural and functional consequences of their binding.

In this work, the interactions of YOYO-1 and YO-PRO-1 with nucleic acids were investigated, with particular attention paid to reaction mechanisms, equilibrium constants, molecular brightnesses, and the influence of temperature. The bis-intercalating dye YOYO-1 was shown to undergo a two-step reaction with DNA, corresponding to the formation of mono- and bis-intercalated complexes. For YO-PRO-1, the mechanism of intercalation into DNA was confirmed. In addition, the interaction between YO-PRO-1 and tRNA was studied, extending the analysis beyond DNA and enabling comparison across different nucleic acid targets.

The results show that, at (25°C), the equilibrium constant for YO-PRO-1 binding to DNA was approximately: $K = (1.1 \pm 0.4) \times 10^6, M^{-1}$

At (36°C), the equilibrium constant for YO-PRO-1 binding to DNA decreased to: $K = (7.5 \pm 2.0) \times 10^5, M^{-1}$

A similar order of magnitude was observed for YO-PRO-1 binding to tRNA at (36°C), with: $K = (7.5 \pm 0.4) \times 10^5, M^{-1}$

These values indicate that YO-PRO-1 forms stable complexes with both DNA and tRNA, and that the affinity toward these two nucleic acid substrates remains within the same order of magnitude under comparable temperature conditions.

The molecular brightness values reveal more pronounced differences between the complexes. At (36°C), the average molecular brightness of the YO-PRO-1–DNA complex was: $\gamma = 6.3 \pm 0.8 \text{ kcnts mol}^{-1}\text{s}^{-1}$ whereas for the YO-PRO-1–tRNA complex it was: $\gamma = 16.2 \pm 1.3 \text{ kcnts mol}^{-1}\text{s}^{-1}$

Thus, the molecular brightness of the YO-PRO-1–tRNA complex was almost three times higher than that of the corresponding DNA complex at the same temperature. This difference suggests that molecular brightness is not determined solely by binding affinity but is also strongly influenced by the local environment of the dye, the nucleic acid conformation, and the rotational and structural mobility of the complex.

A general decrease in equilibrium constants was observed at (36°C) compared with (25°C), suggesting that increased temperature weakens most complex formations. This is consistent with the idea that higher temperature may reduce complex stability by increasing molecular motion and promoting partial destabilisation of non-covalent interactions. Interestingly, the mono-intercalated YOYO-1 complex appeared relatively stable upon temperature change, indicating that this binding state may be less sensitive to thermal effects than other complexes.

YO-PRO-1 displayed an intermediate binding behaviour when compared with the mono- and bis-intercalated YOYO-1 complexes. This may reflect its structural differences from YOYO-1, particularly that YO-PRO-1 is a monomeric dye, whereas YOYO-1 is a bisintercalator capable of engaging DNA via two intercalating units. As a result, YO-PRO-1 may form complexes that are less constrained than bis-intercalated YOYO-1 complexes, but still sufficiently stable to produce strong fluorescence enhancement.

Overall, the obtained results demonstrate that the observed fluorescence signal is governed not only by equilibrium binding strength, but also by reaction mechanism, complex geometry, nucleic acid structure, and molecular mobility.

The main conclusions of this work can be summarised as follows:

1. **YOYO-1 interacts with DNA through a two-step reaction mechanism.** The results support the formation of distinct YOYO-1–DNA complexes, most likely corresponding to mono- and bis-intercalated forms. This confirms that YOYO-1 binding to DNA cannot be fully described as a single-step process.
2. **YO-PRO-1 intercalates into DNA and forms stable complexes.** The determined equilibrium constants confirm that YO-PRO-1 has a high affinity for DNA. At (25°C), the average equilibrium constant was approximately ($1.0 \times 10^6 \text{ M}^{-1}$), while at (36°C) it decreased to approximately ($3.9 \times 10^5 \text{ M}^{-1}$).
3. **YO-PRO-1 also forms stable complexes with tRNA.** The equilibrium constant for the YO-PRO-1–tRNA complex at (36°C) was approximately ($7.5 \times 10^5 \text{ M}^{-1}$), remaining in the same order of magnitude as the YO-PRO-1–DNA interaction. This indicates that YO-PRO-1 may interact efficiently not only with DNA, but also with structured RNA molecules.
4. **Temperature significantly affects complex stability.** For most investigated systems, an increase in temperature from (25°C) to (36°C) caused a decrease in equilibrium constants by approximately one order of magnitude. This suggests that thermal motion weakens the formation of the dye–nucleic acid complex.
5. **The mono-intercalated YOYO-1 complex appears relatively resistant to temperature changes.** In contrast to other complexes, this form showed little variation in its equilibrium constant with temperature. This may indicate a particularly stable intercalation

state or a binding geometry less affected by thermal destabilisation.

6. YO-PRO-1 shows intermediate binding behaviour compared with YOYO-1 complexes. The equilibrium constants of YO-PRO-1–DNA complexes fall between those characteristic of mono- and bis-intercalated YOYO-1 forms. This reflects the different structural organisation of mono- and bis-intercalating dyes.
7. Molecular brightness depends strongly on the type of nucleic acid complex. The YO-PRO-1–tRNA complex displayed nearly threefold higher molecular brightness than the YO-PRO-1–DNA complex at (36°C), despite similar equilibrium constants. Therefore, fluorescence brightness cannot be interpreted as a direct measure of binding affinity alone and is influenced by rotational motion and characteristic time of the rotations.
8. The mobility and structure of the nucleic acid strongly influence the fluorescence response. The higher brightness of the YO-PRO-1–tRNA complex may be related to the compact three-dimensional structure and rotational dynamics of tRNA in the focal volume. This suggests that nucleic acid conformation and molecular motion are crucial parameters in fluorescence-based analyses.
9. Fluorescent dyes should be treated as active molecular probes rather than passive labels. Since intercalators can alter nucleic acid structure, their use in biological and biophysical studies requires careful interpretation. The dye may influence the system being measured, especially in studies involving DNA or RNA structure, dynamics, transcription, or translation.
10. Equilibrium constants, molecular brightness, and reaction mechanisms must be analysed together. A reliable description of dye–nucleic acid interactions requires combining kinetic, equilibrium, and fluorescence parameters. Considering only fluorescence intensity or only binding constants may lead to incomplete or misleading conclusions.

Future prospects

The results obtained in this work open several possible directions for further research.

First, future studies should focus on a more detailed characterisation of alternative binding modes of YOYO-1 and YO-PRO-1. Early circular dichroism studies suggested that YOYO-1 may interact with DNA not only through classical intercalation but also through additional, secondary binding mechanisms. This possibility remains insufficiently explored. Combining fluorescence methods with circular dichroism, fluorescence anisotropy, single-molecule techniques, and molecular dynamics simulations could help distinguish between intercalation, external binding, groove association, and mixed binding modes.

Second, the temperature dependence of dye–nucleic acid interactions should be investigated in greater detail. The observed decrease in equilibrium constants at (36°C) suggests that complex formation is sensitive to thermal conditions. A broader temperature range would allow thermodynamic parameters, such as enthalpy and entropy changes, to be estimated. This would provide deeper insight into the physical forces stabilising each complex.

Third, the interaction between YO-PRO-1 and RNA warrants further attention. The present results show that YO-PRO-1 binds tRNA with an equilibrium constant comparable to that observed for DNA, while producing a much higher molecular brightness. This indicates that structured RNA molecules may be important and previously underestimated targets of cyanine dyes. Future studies should include other RNA types, such as mRNA, rRNA, miRNA, and structured regulatory RNA motifs, to determine whether YO-PRO-1 shows sequence or structural selectivity for RNA.

Fourth, the biological consequences of dye binding should be examined more directly. Since intercalation can modify nucleic acid structure, it may affect DNA replication, transcription, RNA folding, ribosome progression, and translation efficiency. In particular, ligand-induced changes in RNA structure may influence protein production by altering ribosome pausing, elongation dynamics, or accessibility of regulatory regions. Therefore, future work could investigate whether YO-PRO-1 or structurally related intercalators interfere with transcription or translation in model biological systems.

Fifth, the relationship between molecular brightness and molecular mobility should be further clarified. The large difference in brightness between YO-PRO-1–DNA and YO-PRO-1–tRNA complexes suggests that fluorescence intensity is strongly affected by local molecular environment and rotational dynamics. Fluorescence correlation spectroscopy, time-resolved fluorescence, and anisotropy measurements could help determine how molecular rotation, complex compactness, and dye orientation influence the detected signal.

Sixth, the findings may have broader relevance for interpreting fluorescence-based biological assays. YO-PRO-1 and YOYO-1 are commonly used in cell biology, yet their exact intracellular targets and binding mechanisms remain incompletely understood. If these dyes interact not only with DNA but also with RNA or other cellular components, then some experimental results based on their fluorescence signal may require reinterpretation. Future cellular studies should therefore evaluate dye localisation, target specificity, and possible perturbation of nucleic acid-dependent processes.

Finally, the results obtained may serve as a foundation for the design of improved fluorescent probes. A better understanding of how molecular structure, intercalation mechanism, temperature, and nucleic acid mobility affect fluorescence properties could support the development of dyes with higher specificity, reduced structural perturbation, and more predictable photophysical behaviour. Such probes would be valuable not only for basic studies of nucleic acids, but also for diagnostics, imaging, drug discovery, and the investigation of nucleic acid-targeting therapeutics, including intercalating drugs such as anthracyclines.

In conclusion, this work demonstrates that the interactions between cyanine dyes and nucleic acids are complex and strongly dependent on reaction mechanism,

temperature, molecular structure, and mobility. YO-PRO-1 and YOYO-1 should therefore be considered not merely as fluorescent markers, but as active molecular probes whose binding properties can reveal important information about nucleic acid structure and dynamics, while also potentially influencing the biological systems under investigation.

Anthracyclines equilibrium constants

Anthracyclines are an important class of chemotherapeutic agents whose biological activity is strongly associated with their interaction with DNA. Their intercalation into the DNA double helix disrupts DNA structure and interferes with transcription, potentially leading to cell death via apoptosis. Therefore, understanding the physicochemical properties of anthracycline–DNA complex formation is essential not only for describing their molecular behaviour, but also for improving the interpretation of their therapeutic activity.

In this work, the interactions of four clinically relevant anthracyclines with DNA were investigated at 36°C: doxorubicin, daunorubicin, idarubicin, and epirubicin. The obtained equilibrium constants and molecular brightnesses were compared with literature data reported for 25°C by Ying et al. [188]. Thus, the values obtained at 25°C were not measured within the present experimental series, but were used as reference data for assessing the possible influence of temperature on anthracycline–DNA interactions.

The analysed data indicate that the interaction between anthracyclines and DNA cannot be fully described as a simple one-step process. Instead, the results are consistent with a two-step binding model involving the formation of a primary DRUG–DNA complex, followed by a secondary binding event leading to the formation of a (DNA-DRUG)-DRUG complex. The first step may involve the direct intercalation of the anthracycline molecule into the DNA helix, whereas the second step may correspond to the additional association of a drug molecule with the pre-formed complex.

The comparison with the literature data reported at 25°C [188] shows that the equilibrium constants for the formation of the primary DRUG–DNA complexes were lower at 36°C. In general, the decrease was approximately one order of magnitude. This suggests that increased temperature may reduce the apparent affinity of anthracyclines for DNA, possibly due to enhanced molecular motion, altered DNA conformational dynamics, or partial destabilisation of favourable binding interactions.

A stronger temperature-related difference was observed for the secondary (DNA-DRUG)-DRUG complexes. For most of the studied anthracyclines, the equilibrium constants determined at 36°C were lower than the literature values reported at 25°C by approximately two orders of magnitude. This indicates that the secondary binding event is more temperature-sensitive than the primary DRUG–DNA complex formation. Daunorubicin was an exception, since the decrease in the equilibrium constant for its secondary complex was less pronounced, approximately one order of magnitude.

The molecular brightness values also showed compound-specific differences in temperature-related behaviour. In general, increased temperature tended to decrease the molecular brightness of the studied complexes. However, this trend was not universal. Doxorubicin and epirubicin showed higher molecular brightness va-

lues at 36°C than those reported at 25°C, whereas daunorubicin and idarubicin showed lower values. This variability indicates that the fluorescence response is not solely governed by binding affinity. It also depends on the chemical structure of the intercalator, the local environment of the fluorophore, the geometry of the complex, and the mobility of the molecules involved.

Overall, the results show that anthracycline–DNA interactions are temperature-dependent and that different anthracyclines, despite belonging to the same chemical family, exhibit distinct binding and fluorescence behaviour. These findings are important for interpreting fluorescence-based studies, especially in biological systems where temperature can affect both complex formation and the detected fluorescence signal.

Based on the obtained results and their comparison with literature data, the following conclusions can be drawn:

1. The studied anthracyclines, namely doxorubicin, daunorubicin, idarubicin, and epirubicin, form stable complexes with DNA at 36°C.
2. The obtained data are consistent with a two-step model of anthracycline–DNA interaction, involving the formation of a primary DRUG–DNA complex and a secondary ((DNA–DRUG)–DRUG) complex.
3. The primary DRUG–DNA complex is more stable than the secondary complex. This indicates that direct interaction with DNA, most likely related to intercalation, is the dominant binding event.
4. The comparison with literature data reported for 25°C by Ying et al. [188] suggests that increased temperature decreases the apparent affinity of anthracyclines for DNA.
5. The equilibrium constants for primary DRUG–DNA complex formation obtained at 36°C were lower than the literature values reported at 25°C, generally by approximately one order of magnitude.
6. The secondary ((DNA–DRUG)–DRUG) complexes appear to be more temperature-sensitive than the primary complexes. For most anthracyclines, their equilibrium constants decreased by approximately two orders of magnitude compared with the literature values reported at 25°C.
7. Daunorubicin showed a different behaviour from the other studied anthracyclines. The decrease in the equilibrium constant for its secondary complex was less pronounced, suggesting that this complex may have different structural or dynamic properties.
8. Molecular brightness is not directly proportional to binding affinity. Therefore, fluorescence intensity should not be interpreted solely as a measure of complex stability.

9. The effect of temperature on molecular brightness is compound-specific. Doxorubicin and epirubicin showed increased molecular brightness at 36°C compared with the literature data for 25°C, while daunorubicin and idarubicin showed decreased molecular brightness.
10. The comparison with literature data should be interpreted with caution, since differences between the present results and the data reported by Ying et al. [188] may result not only from temperature, but also from differences in experimental conditions, sample preparation, instrumental setup, and data analysis procedures.
11. Temperature should be considered an important factor in fluorescence-based studies of anthracycline–DNA interactions, especially when experimental results obtained under different thermal conditions are compared.
12. The results emphasise that anthracyclines should not be treated as a uniform group of intercalators. Although they share a common chemical background, individual compounds differ in their binding affinity, temperature sensitivity, and fluorescence response.

Future Prospects

The results obtained in this work provide several possible directions for further research. First, future studies should include measurements performed under identical experimental and analytical conditions at several temperatures, including 25°C, 36°C, and additional intermediate temperature points. This would allow the temperature dependence of anthracycline–DNA complex formation to be determined directly, rather than inferred from comparison with literature data. Such an approach would reduce uncertainty related to differences in experimental setup, sample preparation, and fitting procedures.

Second, extending the measurements over a broader temperature range would allow a more detailed thermodynamic analysis of the studied interactions. Determination of parameters such as enthalpy, entropy, and Gibbs free energy changes could help explain why the secondary (DNA-DRUG)-DRUG complexes appear to be more temperature-sensitive than the primary DRUG–DNA complexes. It could also help clarify why daunorubicin shows a different temperature response compared with the other anthracyclines.

Further studies should also focus on the structural origin of the observed differences in molecular brightness. Since the temperature-related changes in brightness were compound-specific, additional experimental techniques would be useful to determine whether these effects arise from altered dye orientation, changes in DNA conformation, solvent accessibility, the fluorophore’s electronic properties, or differences in molecular mobility. Methods such as fluorescence anisotropy, time-resolved fluorescence, circular dichroism, and isothermal titration calorimetry could provide complementary information about the structure and dynamics of the formed complexes.

Another important direction would be to compare anthracyclines with other classes of DNA intercalators. Such studies could help determine whether the observed two-step binding mechanism and temperature sensitivity are general features of intercalator–DNA interactions or specific properties of anthracycline compounds. This would contribute to a broader understanding of how intercalating molecules interact with DNA and how their chemical structure affects complex formation.

The biological relevance of temperature-dependent binding should also be explored in more complex systems. In cells, anthracycline–DNA interactions occur under physiological conditions, where temperature, ionic strength, molecular crowding, chromatin organisation, and the presence of proteins may affect complex formation. Therefore, future studies performed in cellular or cell-like environments could provide a more realistic description of anthracycline behaviour *in vivo*.

Particular attention should also be paid to the possible consequences of secondary complex formation. If additional anthracycline molecules can associate with pre-formed DRUG–DNA complexes, this may influence local drug accumulation, DNA structural deformation, transcription inhibition, and cytotoxicity. A better understanding of this process could contribute to a deeper explanation of anthracycline activity and its side effects.

Finally, the obtained results may support the development of improved fluorescence-based assays and more effective DNA-targeting compounds. Since molecular brightness depends on temperature and varies among anthracyclines, fluorescence intensity should be interpreted carefully, especially in experiments conducted under variable thermal conditions. Including temperature calibration and complementary structural analysis may improve the reliability of fluorescence-based measurements.

In conclusion, this work demonstrates that anthracycline–DNA interactions are complex, multi-step, and sensitive to temperature. The comparison with literature data [188] suggests that increased temperature may reduce the stability of the formed complexes and affect fluorescence properties in a compound-specific manner. These findings are important for interpreting fluorescence-based studies and may contribute to a better understanding of the molecular mechanisms underlying anthracycline activity.

Fraction of emitting YO-PRO-1 complexes with immobilised DNA

The structure of DNA in the cell nucleus may be considered nearly immobile on the time scale relevant for many fluorescence experiments. This immobilisation can significantly influence the fluorescence signal, especially when fluorescent molecules are intercalated into DNA and therefore fixed in specific orientations relative to the excitation laser beam. Since the probability of excitation depends on the relative orientation between the transition dipole moment of the fluorophore and the electric field vector of the incident light, not all immobilised fluorophores contribute equally to the detected fluorescence signal.

In this work, a simplified model of immobilised naked DNA was used. This approach allowed the photobleaching behaviour of DNA–YO-PRO-1 complexes to be studied under controlled conditions, while excluding the additional complexity present in the cell nucleus. Therefore, effects related to chromatin organisation, DNA binding site accessibility, transcriptional activity, and the presence of DNA-

associated proteins or other molecules were not considered in the experimental model.

The analysis was focused on the influence of laser polarisation and temperature on the number of photobleached DNA–YO-PRO-1 complexes. The experiments were performed using linearly and circularly polarised laser beams at 25°C and 36°C. The calculated number of photobleached molecules was compared with the theoretical number of visible complexes in the focal volume.

The obtained data show that a larger number of molecules was photobleached at 36°C than at 25°C. This effect may be explained by the larger focal volume at higher temperatures. An increase in the focal volume naturally leads to a higher theoretical number of DNA–YO-PRO-1 complexes located within the region of interest, thereby increasing the number of molecules that can potentially undergo photobleaching.

However, the percentage of photobleached complexes did not change substantially with temperature. For linear polarisation, the percentage increased only slightly from approximately 60% at 25°C to approximately 61% at 36°C. For circular polarisation, the corresponding values were approximately 49% and 52%. This suggests that although the absolute number of photobleached molecules increases with temperature, the relative fraction of photobleached complexes remains similar.

A clear difference was observed between the two laser polarisation modes. In both temperature conditions, linear polarisation resulted in a higher percentage of photobleached complexes than circular polarisation. The difference was approximately 10%, indicating that linearly polarised light photobleached a larger fraction of immobilised DNA–YO-PRO-1 complexes. This can be explained by the more directional distribution of the electric field in the case of linear polarisation, which enables more efficient excitation of favourably oriented fluorophores. In contrast, circular polarisation excites molecules over a broader range of orientations, but with less concentrated excitation efficiency in any single direction.

The results indicate that approximately 50–60% of immobilised DNA–YO-PRO-1 complexes were photobleached during the measurements, whereas approximately 40–50% of the complexes remained non-excited or insufficiently excited to undergo photobleaching. This observation is particularly important for fluorescence studies of immobilised systems. It shows that a considerable fraction of fluorophores may remain optically inactive under given excitation conditions, most likely due to unfavourable molecular orientation or limited excitation efficiency.

Overall, these findings demonstrate that photobleaching in immobilised DNA–dye systems is governed not only by the number of molecules in the focal volume, but also by the polarisation of the excitation beam, the orientation of fluorophores, temperature-dependent changes in focal volume, and molecular brightness. These factors must be considered when interpreting fluorescence-based measurements of immobilised nucleic acid complexes.

Based on the obtained results, the following conclusions can be drawn:

- 1. The immobilisation of DNA–YO-PRO-1 complexes strongly influences their fluorescence behaviour, because the excitation effi-**

ciency depends on the orientation of the fluorophore relative to the laser beam.

2. The simplified model of naked immobilised DNA allowed the influence of laser polarisation and temperature on photobleaching to be analysed without additional effects related to chromatin structure, transcription, DNA-associated proteins, or limited accessibility of binding sites.
3. A higher number of photobleached complexes was observed at 36°C than at 25°C. This effect can be attributed mainly to the larger focal volume at higher temperature, which increases the theoretical number of DNA–YO-PRO-1 complexes present in the region of interest.
4. Despite the higher absolute number of photobleached molecules at 36°C, the percentage of photobleached complexes remained relatively similar at both temperatures.
5. For linear polarisation, the percentage of photobleached complexes was approximately $59.97 \pm 16.27\%$ at 25°C and $61.15 \pm 19.26\%$ at 36°C.
6. For circular polarisation, the percentage of photobleached complexes was lower, reaching $49.29 \pm 15.58\%$ at 25°C and $51.89 \pm 16.04\%$ at 36°C.
7. Linear polarisation resulted in a higher fraction of photobleached complexes than circular polarisation. The difference between the two polarisation modes was approximately 10%.
8. The higher photobleaching efficiency observed for linear polarisation may result from the more directional and concentrated distribution of excitation energy, which favours efficient excitation of fluorophores aligned with the electric field vector.
9. Circular polarisation may excite a broader range of molecular orientations, but with lower excitation efficiency for individual immobilised molecules, resulting in a lower percentage of photobleached complexes.
10. Approximately 50 – 60% of immobilised DNA–YO-PRO-1 complexes were photobleached, while approximately 40 – 50% of complexes did not contribute effectively to the photobleaching signal.
11. The presence of a large non-photobleached fraction indicates that a significant number of immobilised fluorophores may remain optically inactive during fluorescence measurements due to unfavourable orientation or insufficient excitation.

12. **The results emphasise that fluorescence intensity and photobleaching data obtained for immobilised systems cannot be interpreted solely as a direct measure of the total number of fluorophores.**
13. **In immobilised fluorescence experiments, laser polarisation, focal volume, temperature, molecular brightness, and fluorophore orientation must be considered together, because their combined effects determine the observed signal.**

Future prospects

The obtained results indicate several important directions for future research. First, further studies should include a more detailed analysis of the orientation dependence of excitation and photobleaching in immobilised DNA–dye complexes. Since a substantial fraction of complexes remained non-photobleached, it would be valuable to determine how the orientation of YO-PRO-1 molecules within the DNA helix affects their probability of excitation. This could be achieved by combining photobleaching experiments with fluorescence anisotropy or polarisation-resolved fluorescence measurements.

Second, future experiments should investigate a broader range of polarisation conditions. In this work, linear and circular polarisations were compared, but intermediate elliptical polarisations could provide additional insight into how the spatial distribution of the electric field affects excitation efficiency. Such measurements would help establish whether the fraction of optically inactive molecules can be reduced by optimising the polarisation state of the excitation beam.

Third, the temperature dependence of photobleaching should be studied in greater detail. Although the present results show that the absolute number of photobleached molecules increases at 36°C, the relative percentage of photobleached complexes remains similar. Measurements at additional temperatures would allow the contributions of focal volume, molecular brightness, diffusion-related effects, and photobleaching efficiency to be separated more precisely.

Further studies should also include more complex DNA models. The present work used naked immobilised DNA, which allowed the basic physical effects to be isolated. However, DNA in the cell nucleus is associated with histones and other proteins, organised into chromatin, and involved in dynamic biological processes such as transcription and replication. Therefore, future experiments using chromatin-like systems or cell-based models would help determine how nuclear organisation affects the accessibility, orientation, and fluorescence response of intercalated dyes.

Another important direction would be the development of correction factors for fluorescence-based quantification in immobilised systems. Since approximately 40–50% of complexes may remain non-excited under the applied conditions, standard fluorescence measurements may underestimate the actual number of fluorophores. Introducing calibration procedures that account for the non-emitting or weakly excited fraction would improve the accuracy of quantitative fluorescence studies.

The results are also relevant for Fluorescence Correlation Spectroscopy and other fluorescence-based techniques applied to immobilised or partially immobilised samples. In such systems, the detected fluorescence signal may originate only from a subset of favourably oriented molecules. Future methodological work

should therefore consider how molecular orientation and laser polarisation influence apparent molecular brightness, concentration estimates, and photobleaching kinetics.

Finally, future studies should combine experimental data with theoretical modelling of the excitation field and fluorophore orientation. Simulations of the electric field distribution in the focal volume, together with models of dye orientation within DNA, could help explain why only a fraction of immobilised complexes is efficiently excited. Such an approach would provide a more complete description of fluorescence excitation and photobleaching in immobilised nucleic acid systems.

In conclusion, this work demonstrates that photobleaching of immobilised DNA–YO-PRO-1 complexes is controlled by a complex interplay between temperature, focal volume, laser polarisation, molecular brightness, and fluorophore orientation. The finding that only approximately 50–60% of immobilised complexes contribute effectively to the photobleaching signal is particularly important for the interpretation of fluorescence-based studies. These results highlight the need for careful calibration and methodological awareness when analysing immobilised molecular systems.

YO-PRO-1 cellular targets

YO-PRO-1 is commonly used as an early apoptosis indicator and is often considered a cell-impermeant dye. However, the results obtained in this work do not fully support this commonly accepted interpretation. In the performed cellular experiments, YO-PRO-1 entered HeLa cells and was not selectively associated with apoptotic cells. Moreover, the dye did not primarily stain nuclear DNA under the experimental conditions used. Instead, the obtained results indicate that YO-PRO-1 interacts with RNA-containing cellular structures.

The cellular experiments showed that YO-PRO-1 produced fluorescence signals after incubation with HeLa cells, suggesting that the dye entered the cellular interior and interacted with intracellular components. Further analysis indicated that the observed fluorescence was not mainly associated with nuclear DNA. Instead, the dye showed affinity toward RNA-rich targets, particularly rRNA and tRNA. This observation changes the interpretation of YO-PRO-1 staining in cells and suggests that the dye should not be treated only as a marker of apoptosis or DNA accessibility.

The interaction of YO-PRO-1 with cellular lysate components resulted in an increase in fluorescence intensity and autocorrelation signal. However, the autocorrelation curves for lysate samples could not be reliably fitted due to the sample's complexity and the presence of numerous fluorescent species. This indicates that the lysate contained multiple YO-PRO-1-binding components of different sizes and diffusion properties. Therefore, although the lysate experiment confirmed that YO-PRO-1 interacts with intracellular molecules, it did not allow individual targets to be separated quantitatively.

To further examine the role of RNA in YO-PRO-1 binding, RNase-treated samples were analysed. In these samples, the increase in fluorescence count rate was lower than in the untreated lysate. This result supports the conclusion that RNA contributes significantly to YO-PRO-1 fluorescence in the cellular environment. RNase digestion cleaves RNA molecules into shorter fragments, thereby altering the number, size, and diffusion properties of fluorescent species within

the focal volume. The reduced fluorescence response after RNase treatment indicates that intact RNA-containing structures are important targets of YO-PRO-1.

As an extension of these observations, the total RNA pool was studied in the presence of YO-PRO-1. The obtained data were described using a two-component diffusion model with rotational diffusion of the larger component. The first component was characterised by a hydrodynamic radius of approximately 2.1 nm, a diffusion coefficient of $153 \mu\text{m}^2/\text{s}$, and a concentration of 5.3 nM. These parameters are consistent with the expected size and diffusion behaviour of tRNA. The second component was substantially larger, with a hydrodynamic radius of approximately 24.5 nm, a diffusion coefficient of $12.1 \mu\text{m}^2/\text{s}$, and a concentration of 8.5 nM. These values suggest that this component may correspond to ribosomal particles containing rRNA and ribosomal proteins. This supports the conclusion that YO-PRO-1 interacts not only with DNA but also with RNA-containing cellular structures, including tRNA and ribosomes.

The interaction between YO-PRO-1 and tRNA was further confirmed in measurements performed on a tRNA–YO-PRO-1 mixture. The data could be fit with a one-component diffusion model, consistent with the presence of a single dominant fluorescent species. The calculated hydrodynamic radius was approximately 1.9 nm, the diffusion coefficient was $167 \mu\text{m}^2/\text{s}$, and the concentration was 39.3 nM. These values are in good agreement with the expected properties of tRNA and confirm that tRNA is a cellular target of YO-PRO-1.

The study also identified the large ribosomal subunit as another potential cellular target of YO-PRO-1. The analysis of translational and rotational diffusion of the large ribosomal subunit further supported the interaction of YO-PRO-1 with RNA-rich ribosomal structures. This observation is particularly important because ribosomes contain rRNA as a major structural and functional component. Therefore, YO-PRO-1 staining may reflect interactions with ribosomal RNA and ribosomal particles rather than only with DNA or apoptotic cells.

Overall, the obtained results indicate that YO-PRO-1 has a broader range of cellular targets than is commonly assumed. The dye can enter cells, interact with RNA-containing structures, and bind to tRNA, rRNA, and ribosomal subunits. These findings contribute to a more complete understanding of YO-PRO-1 behaviour in biological systems and suggest that its cellular fluorescence signal should be interpreted with caution.

Based on the obtained results, the following conclusions can be drawn:

- 1. YO-PRO-1 should not be considered exclusively as an early apoptosis indicator under all experimental conditions.**
- 2. In the performed experiments, YO-PRO-1 was able to enter HeLa cells, which does not support the assumption that the dye is strictly cell-impermeant.**
- 3. YO-PRO-1 staining was not primarily associated with nuclear DNA in the studied cellular system.**
- 4. The obtained results indicate that YO-PRO-1 interacts with RNA-containing cellular components.**

5. RNase treatment reduced the fluorescence response compared with untreated lysate, supporting the conclusion that RNA contributes significantly to the YO-PRO-1 fluorescence signal.
6. The complexity of the cellular lysate prevented reliable fitting of autocorrelation curves, indicating the presence of multiple fluorescent species with different diffusion properties.
7. Measurements of the total RNA pool revealed two main fluorescent components in the presence of YO-PRO-1.
8. The smaller component, characterised by a hydrodynamic radius of approximately 2.1 nm and a diffusion coefficient of $153 \mu\text{m}^2/\text{s}$, was consistent with tRNA.
9. The larger component, characterised by a hydrodynamic radius of approximately 24.5 nm and a diffusion coefficient of $12.1 \mu\text{m}^2/\text{s}$, was consistent with ribosomal particles containing rRNA and ribosomal proteins.
10. Direct measurements of the tRNA–YO-PRO-1 mixture confirmed that tRNA is a target of YO-PRO-1.
11. The hydrodynamic parameters obtained for the tRNA–YO-PRO-1 complex, including a radius of approximately 1.9 nm and a diffusion coefficient of $167 \mu\text{m}^2/\text{s}$, are consistent with the expected properties of tRNA.
12. The large ribosomal subunit was identified as another potential cellular target of YO-PRO-1.
13. The interaction of YO-PRO-1 with rRNA and ribosomal structures suggests that the dye may be useful in studies of ribosomes, the large ribosomal subunit, and nucleoli.
14. Since rRNA biosynthesis takes place in nucleoli, YO-PRO-1 may potentially be developed as a tool for studying nucleolar RNA-related processes.
15. The cellular fluorescence signal of YO-PRO-1 should be interpreted carefully, because it may originate from RNA-containing structures rather than from apoptotic cells or nuclear DNA alone.

Future prospects

The results obtained in this work open several possible directions for further research. First, the cellular targets of YO-PRO-1 should be investigated in greater detail using more selective experimental approaches. Since the dye was shown to interact with tRNA, rRNA, and ribosomal structures, future studies should aim to determine the relative contributions of these targets to the total cellular fluorescence signal. This could help clarify whether YO-PRO-1 preferentially binds

specific RNA structures or whether its interaction is more generally dependent on RNA folding, charge distribution, and accessibility.

Second, further studies should focus on the interaction between YO-PRO-1 and ribosomal particles. The identification of the large ribosomal subunit as a potential target suggests that YO-PRO-1 may be useful for studying ribosome-related processes. Future experiments could compare the binding of YO-PRO-1 to isolated ribosomal subunits, complete ribosomes, rRNA fragments, and ribosomal protein–RNA complexes. Such studies would help determine whether the dye interacts mainly with rRNA or whether ribosomal proteins also influence binding.

Third, YO-PRO-1 may be further developed as a tool for studying nucleoli. Since nucleoli are the cellular compartments where rRNA biosynthesis and ribosome assembly take place, the interaction of YO-PRO-1 with rRNA and ribosomal components may provide a basis for nucleolar imaging or for monitoring changes in rRNA-related processes. Future cell-based experiments should therefore examine the intracellular localisation of YO-PRO-1 in relation to nucleolar markers.

Another important direction would be to re-evaluate the use of YO-PRO-1 as an apoptosis marker. The observation that YO-PRO-1 can enter cells and interact with RNA-containing structures suggests that its fluorescence signal may not be specific to apoptotic cells. Future studies should compare YO-PRO-1 staining with independent apoptosis markers, membrane integrity assays, and RNA-specific dyes. This would help determine under which conditions YO-PRO-1 can be reliably used as an apoptosis indicator and when its signal may reflect other cellular processes.

Further research should also investigate the mechanism by which YO-PRO-1 enters cells. Since the dye is commonly considered cell-impermeant, understanding its cellular uptake is essential for interpreting staining experiments. Possible mechanisms may include passive entry under specific conditions, transport through membrane defects, endocytosis, or changes in membrane permeability unrelated to apoptosis. Clarifying this process would improve the reliability of YO-PRO-1-based cellular assays.

The interaction between YO-PRO-1 and RNA should also be studied using complementary biophysical techniques. Fluorescence correlation spectroscopy can provide valuable information about diffusion and molecular size, but additional methods such as fluorescence anisotropy, time-resolved fluorescence, circular dichroism, electrophoretic mobility assays, and structural modelling could help describe the binding mechanism more precisely. These approaches would enable determination of whether YO-PRO-1 binds RNA via intercalation, groove-like association, electrostatic interactions, or a mixed binding mode.

Future studies should also include different RNA species, including mRNA, rRNA fragments, structured regulatory RNAs, and synthetic RNA models. Comparing these targets would help determine whether YO-PRO-1 displays selectivity toward specific RNA structures. This is particularly important because RNA molecules differ strongly in size, folding, base stacking, and accessibility, all of which may influence dye binding and fluorescence enhancement.

Finally, the findings of this work should be considered in the broader context of fluorescence-based cellular studies. If YO-PRO-1 binds RNA-rich structures and ribosomal components, then fluorescence signals obtained in cells may require re-

interpretation, especially in experiments where the dye is assumed to indicate only apoptosis or DNA staining. Future applications of YO-PRO-1 should therefore include appropriate controls, such as RNase and DNase treatments, co-localisation with RNA and nucleolar markers, and comparison with established apoptosis assays.

In conclusion, this work demonstrates that YO-PRO-1 has important cellular targets beyond DNA and apoptotic cells. The dye interacts with tRNA, rRNA, and ribosomal structures, including the large ribosomal subunit. These findings expand the understanding of YO-PRO-1 behaviour in biological systems and suggest that the dye may be useful not only as a cellular stain, but also as a potential probe for RNA-rich structures, ribosomal components, and nucleolar processes.

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